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# REGULATION OF EXTRACELLULAR SIGNAL-REGULATED PROTEIN KINASES (ERK) 1 & 2 IN SYNAPTIC NERVE TERMINALS

By

Vivian Wing Yan Lee

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To my Heavenly Father

and my family with Love

**DECLARATION**

I, Vivian Wing Yan Lee, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Vivian W. Y. Lee

## ABSTRACT

Activation of extracellular signal-regulated kinases 1&2 (ERK 1&2) is a key signalling event in the modulation of presynaptic function. This study looks at the upstream regulation of ERK 1&2 signalling in rat cerebrocortical synaptosomes. Kinase activation assays based on phospho-state specific antibodies revealed two major inputs for the activation of ERK 1&2: i) a neurotrophin-mediated signalling to ERK 1&2 which is dependent on the activation of small GTP-binding protein Ras and the presence of $Ca^{2+}$; ii) a $Ca^{2+}$-dependent activation of ERK 1&2, stimulated by depolarisation or by the $Ca^{2+}$ ionophore ionomycin. Treatment of synaptosomes with $Ca^{2+}$ chelators showed that basal ERK 1&2 activation was partly supported by $Ca^{2+}$ from intracellular sources, whilst depolarisation-dependent activation of ERK 1&2 was entirely attributable to extracellular $Ca^{2+}$ influx. Like the BDNF-mediated activation, this $Ca^{2+}$-dependent signalling to ERK was contingent on Ras, as evinced by the use of Ras inhibitor lovastatin. Using inhibitors of $Ca^{2+}$/CaM-dependent protein kinase II (CaMKII) and phosphatidylinositol-3-kinase (PI3K), we next showed that both kinases are involved in mediating the $Ca^{2+}$-dependent ERK 1&2 pathway. Furthermore, the Src family kinase (SFK) inhibitor, PP2, strongly reduced $Ca^{2+}$-dependent ERK 1&2 activation, suggesting a role for non-receptor tyrosine kinases (nRTKs) in upstream signalling. Finally, using okadaic acid, roscovitine and baclofen respectively, we showed that the duration and efficacy of ERK 1&2 activation are determined by the function of protein phosphatase 2A (PP2A), cyclin-dependent kinase 5 (cdk5) and the presynaptic $G_{i/o}$-coupled $GABA_B$ receptors. Interestingly, our data demonstrate that baclofen-mediated inhibition of ERK 1&2 can be overridden by BDNF stimulation, revealing a potential feedback and cross-talk mechanism between the excitatory ERK and inhibitory GABA cascades. Together, these studies elucidate the role of ERK 1&2 as a hub for signalling in the nerve terminal.

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## ABBREVIATIONS

| | |
|---|---|
| AC | Adenylyl cyclase |
| AMPA | α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid |
| 4AP | 4-aminopyridine |
| ATP | Adenosine 5'-triphosphate |
| BAPTA-AM | bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid |
| BDNF | Brain-derived neurotrophic factor |
| BSA | Bovine serum albumin |
| $[Ca^{2+}]_c$ | Cytosolic free $Ca^{2+}$ concentration |
| CaM | Calmodulin |
| CaMKI and II | $Ca^{2+}$/calmodulin-dependent protein kinase I and II |
| cAMP | Adenosine 3'-5'-cyclic monophosphate |
| Cdk5 | Cyclin-dependent protein kinase 5 |
| CNS | Central nervous system |
| DAG | Diacylglycerol |
| DMSO | Dimethyl sulfoxide |
| EGF | Epidermal growth factor |
| EGTA | Ethylene glycolbis(β-aminoethyl ether)-N,N,N,N'-tetraacetic acid |
| EPSP | Excitatory postsynaptic potential |
| ERK 1 and 2 | Extracellular-signal-regulated kinase 1 and 2 |
| FAK | Focal adhesion kinase |
| Fura-2-AM | Fura-2 acetoxymethylester |
| GABA | γ-aminobutyric acid |
| GAP | GTPase activating protein |
| GBD | GTP-binding domain |
| GDH | Glutamate dehydrogenase |
| GDP | Guanosine diphosphate |
| GEF | Guanine nucleotide exchange factor |
| GHB | γ-hydroxbutyrate |
| GPCR | G-protein coupled receptor |
| Grb2 | Growth factor-receptor-bound protein |
| GRF | Guanine nucleotide-releasing factor |
| GTP | Guanosine triphosphate |
| HBM | Hepes buffered medium |
| Hepes | N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid |
| HPLC | High performance liquid chromatography |
| $IP_3$ | Inositol-1,4,5-triphosphate |
| IPSP | Inhibitory postsynaptic potential |

| | |
|---|---|
| KCl | Potassium chloride |
| kDa | Kilodaltons |
| KSR | Kinase suppressor of Ras |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| MAPK | Mitogen-activated protein kinase |
| MEK | Mitogen-activated kinase/ERK kinase (MAP kinase kinase) |
| mEPSCs | Mini excitatory post-synaptic currents |
| mGluR | Metabotropic glutamate receptor |
| MKKK | MAPK kinase kinase |
| MKP 1 and 2 | MAP kinase phosphatase 1 and 2 |
| MP1 | MEK partner 1 |
| MW | Molecular weight |
| nAChR | Nicotinic acetylcholine receptor |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate |
| NGF | Nerve growth factor |
| NMDA | N-methyl-D-aspartate |
| nRTK | Non-receptor tyrosine kinase |
| NSF | N-ethylmalemide-sensitive fusion protein |
| PBS | Phosphate buffer saline |
| PI3K | Phosphatidylinositol-3-kinase |
| $PIP_2$ | Phosphatidylinositol 4,5-bisphosphate |
| PKA | cAMP-dependent protein kinase (Protein kinase A) |
| PKB | Protein kinase B / Akt |
| PKC | $Ca^{2+}$/phospholipid-dependent protein kinase (Protein kinase C) |
| PLC | Phospholipase C |
| PP1 | Protein phosphatase 1 |
| PP2A | Protein phosphatase 2A |
| PP2B | Protein phosphatase 2B (calcineurin) |
| PTB | Phosphotyrosine binding domain |
| PTKs | Protein tyrosine kinases |
| PTPs | Protein tyrosine phosphatases |
| Pyk2 | Proline-rich tyrosine kinase 2 |
| RTK | Receptor tyrosine kinases |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEM | Standard error of the mean |
| SERCA | Sacro-endoplasmic reticulum $Ca^{2+}$-ATPase |
| SFK | Src family kinase |
| SH2 | Src-homology 2 domain |
| SH3 | Src-homology 3 domain |

| | |
|---|---|
| Shc | Src/α-collagen |
| SNAP-25 | Synaptosome-associated protein 25 |
| SNAPs | Soluble NSF attachment proteins |
| SNARE | SNAP receptor |
| Sos | Sons of sevenless |
| SV | Synaptic vesicles |
| SynGAP | A GAP for Ras |
| TBS | Tris-buffered saline |
| TBS-T | TBS with Tween-20 |
| TNF | Tumour necrosis factor |
| Trk | Tropomycin-related kinase |
| Tris | Tris (hydroxymethyl)aminoethane |
| t-SNARE | Target-associated SNARE |
| VAMP | Vesicle-associated membrane protein (synaptobrevin) |
| VGCC | Voltage-gated calcium channel |
| v-SNARE | Vesicle-associated SNARE |

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# Chapter 1

# INTRODUCTION

# 1. INTRODUCTION

The human brain is made up of a vast circuit of nerve cells (neurons) that are capable of conveying information in a highly specific manner. Historically, these neurons were seen as a continuous network of nerves. It was not until later on when Ramón y Cajal reported the idea of discontinuity between individual neurons. In his work, Cajal described neurons as individual cells containing 3 parts: dendrites, soma (cell body) and axon that end and formed functional contacts with a neighbouring neuron. This hypothesis of discontinuity was further developed by Charles S. Sherrington, who led to the idea of the synapse or neuronal junction composed of the presynaptic terminal, the postsynaptic terminal and the synaptic cleft (space between the pre- and post-synaptic terminals). And it is this synapse, which is now considered to be crucial for regulating signal transmission between neurons and for the modulation of responses.

In general, a neuronal response is generated when depolarising action potentials arrive at the synapse to trigger a series of molecular events that lead to the release of neurotransmitters into the synaptic cleft by means of exocytosis or emptying of synaptic vesicles (SVs). Once released, these neurotransmitters diffuse across the synaptic cleft to act on receptors to bring about an ionotropic and/or a metabotropic response. As neurons are known to transduce signals with high accuracy and fidelity, the regulation of how a synapse initiates a particular synaptic function and/or release of neurotransmitters following a stimulus is of particular interest to neuroscientists and is the subject of this thesis. Notwithstanding modulation of synaptic function can be exerted post-junctionally with the presence of postsynaptically located receptors, this thesis focuses on the regulatory mechanisms in the presynaptic digit. Increasingly, a number of receptors (autoreceptors and heteroreceptors) have been identified presynaptically to control neuronal function. Elucidation of the molecular signalling pathways and mediators affecting the control of this presynaptic release will provide a better understanding of the precise mechanisms by which neurons

effect a synaptic response.

### 1.1. Synaptic plasticity

During nerve stimulation, synapses undergo changes in their synaptic strength in response to the varied patterns of ongoing activity. These changes occur to increase (facilitate) or decrease (depress) the efficacy of synaptic transmission, bringing about either short-term or long-term alterations. Stimulation with short trains of presynaptic action potentials produce changes in presynaptic functions that last for several hundred milliseconds whereas longer-lasting trains produce increase in the synaptic potential amplitude, known as post-tetanic potentiation (PTP), that may last for one to two minutes. The induction of long-term changes, either in the form of long-term potentiation (LTP) or long-term depression (LTD), can last for hours or even days and depends greatly on the strength of both pre- and post-synaptic action potentials.

Several underlying mechanisms cooperate to achieve synaptic plasticity. These include changes in the amount of neurotransmitter release and the ability of neurons to respond to these neurotransmitters (Gaiarsa et al., 2002). In LTP or LTD, persistent changes may involve the insertion of new receptors into the membrane of the postsynaptic terminals and/or an increase of receptor efficacy (Song and Huganir, 2002). In both short-term and long-term changes, much of the regulation of synaptic plasticity is mediated by changes in intracellular calcium ($Ca^{2+}_i$) in the synaptic nerve terminals. It has been established that facilitation of neurotransmitter release following a train of stimuli was linked to residual $Ca^{2+}$ from the previous action potentials (Katz and Miledi, 1968). Repetitive stimulation would then cause a continued accumulation of $Ca^{2+}$ to result in a progressive increase in neurotransmitter release (Augustine, 2001; Catterall, 2000; Hsu et al., 1996; Heidelberger et al., 1994). The importance of $Ca^{2+}$ in neuronal function has also been shown in studies where $Ca^{2+}$ dysregulation has been implicated in ischaemic neuronal death, excitotoxicity and neuronal diseases such as Alzheimer's disease (Won et al., 2002; Salinska et al.,

2005; Smith et al., 2005).

Stimulation of synaptic function by exogenous factors such as neurotrophins also provide means of regulating synaptic efficacy. Studies have shown that application of brain-derived neurotrophic factor (BDNF) could have both short and long term effect in enhancing synaptic output via the activation of TrkB receptors (Tyler et al., 2002; Jovanovic et al., 2000; Baldelli et al., 2000; Li et al., 1998). BDNF has been suggested to attenuate synaptic fatigue in hippocampal CA1 (Pozzo-Miller et al., 1999), where effects may be mediated via the activation of protein kinases including extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase (PI3K) (Gottschalk et al., 1999).

Another way by which synaptic function could be regulated is through protein phosphorylation, a major post-translational protein modification that determines the activity or function of targets. Thus, protein kinases effect covalent modification of serine, threonine or tyrosine using the high energy γ-phosphate from adenosine triphosphate (ATP) (Engh and Bossemeyer, 2001). Protein phosphatases, on the other hand, reverse this process. Indeed, it is becoming evident that many of the proteins involved in regulating synaptic plasticity (including $Ca^{2+}$ channels, proteins in SV trafficking or in the exocytotic machinery) are subject to regulation by phosphorylation. Unlike $Ca^{2+}$, protein phosphorylation likely plays a modulatory role rather than being directly involved in the process of exocytosis.

One of the objectives of this thesis is to investigate the mechanisms by which presynaptic function could be regulated by $Ca^{2+}$, neurotrophins as well as modulatory processes such as phosphorylation and dephosphorylation. The subsequent introduction, therefore, considers the modulatory influences on presynaptic function, in particular, neurotransmitter release and the role of $Ca^{2+}$, neurotrophins and

phosphorylation underlying the control of presynaptic efficacy.

### 1.2. Modulation of presynaptic function

Control of presynaptic function can take place at any of the stages leading up to exocytosis. Generally, four major events occur following the arrival of action potential at the presynaptic nerve terminal: i) depolarisation of presynaptic membrane due to $Na^+$ and $K^+$ channel activities; ii) $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels (VGCCs); iii) the release of neurotransmitters (exocytosis); iv) the retrieval and further recruitment of SVs to the site of neurotransmitter release.

#### 1.2.1. Depolarisation in the nerve terminal

One of the ways by which presynaptic function could be regulated is through modulating the excitability of plasma membrane. The resting membrane potential is controlled by $K^+$ channels activated between -90 to -70mV. During synaptic transmission, the arrival of action potential activates voltage-dependent $Na^+$ channels to mediate $Na^+$ entry. This causes the presynaptic membrane to become depolarised, thereby activating $Ca^{2+}$ influx through the opening of voltage-gated $Ca^{2+}$ channels (VGCCs) to trigger exocytosis. Once the stimulation is terminated, repolarisation takes place where delayed rectifier $K^+$ channels become activated to restore membrane potentials to resting levels. Thus, by modulating membrane excitability either by altering the activation of $Na^+$ or $K^+$ channel, the subsequent activation of VGCCs and process of exocytosis can also be affected. Studies have shown that neuronal excitability could be modulated by the phosphorylation of $Na^+$ channels by protein kinase A (PKA) (Cantrell et al., 1997; Murphy et al., 1996). In addition, protein kinase C (PKC) has been suggested to phosphorylate $K^+$ channels and, in so doing, destabilise the presynaptic membrane potential to consequently potentiate glutamate release, although this has not been directly tested (Barrie et al., 1991).

### 1.2.2. Modulation of $Ca^{2+}$ influx

Another potential target for the regulation of neurotransmitter release is at the level of $Ca^{2+}$ entry involving the control of VGCC activation. The $Ca^{2+}$ channels that are involved in triggering release are primarily high voltage-activated P/Q and N-type VGCCs, which have been found co-localised to the active zones, membrane regions of the presynaptic terminal juxtaposed to the postsynaptic density (Reid et al., 2003; Catterall, 2000; Bowman et al., 1993). Studies using selective antagonists or toxins (ω-agatoxin IVA and ω-conotoxin GVIA against P/Q type and N-type VGCCs respectively) have demonstrated that blockade of both VGCC subtypes causes a block of synaptic currents which sum up to >100%, suggesting that these VGCCs have overlapping contributions to the local $Ca^{2+}$ concentration to elicit neurotransmitter release (Reid et al., 2003; Reid et al., 1997; Wheeler et al., 1996; Wu and Saggau, 1994). In some synapses, there is evidence that residual current still occurs following the blockade of P/Q and N-type VGCCs. This may be attributable to the R-type VGCCs, channels that are mainly perisynaptically localized but are also found in the active zone. These R-type VGCCs are now considered to be competent to evoke exocytosis, although the contribution is only partial (Kamp et al., 2005; Reid et al., 2003; Gasparini et al., 2001). These R-type channels have been implicated in LTP where mice with genetically ablated R-type VGCCs produce impaired presynaptic LTP and PTP (Dietrich et al., 2003). Interestingly, studies have revealed that the contribution of R-type VGCCs to neurotransmitter release is upregulated in $Ca_v2.1$(P/Q-type VGCC)-deficient mice, suggesting that R-type VGCCs can act to replace P/Q-type functions during conditions when P/Q-type VGCC activities are decreased (Pagani et al., 2004). Furthermore, cooperativity of channel functions was also evident in Purkinje cells where both N-type and R-type VGCC activities were increased in response to antisense-RNA knock-down of P/Q-type VGCCs (Gillard et al., 1997).

In general, the amount of neurotransmitter release depends on the degree of $Ca^{2+}$

influx, the spatial arrangement of VGCC and the presence of $Ca^{2+}$ binding proteins/buffers (Augustine, 2001). In this regard, VGCCs have been found close to the release machinery at the active zone, where the $Ca^{2+}$ sensor synaptotagmin is also available to detect changes in local $Ca^{2+}$ concentration (Augustine, 2001). This is achieved by specific interaction of the channels with members of the SNARE proteins (section 1.2.3) that compose the exocytosis machinery, through the synprint domain in the II-III linker region of $\alpha_1$ of the P/Q and N-type VGCCs (Rettig et al., 1997; Yokoyama et al., 1997). Additionally, there is evidence that cytoskeletal elements may also act to localise VGCCs in the nerve terminal (Spafford and Zamponi, 2003).

During nerve stimulation, depolarisation activates $Ca^{2+}$ influx through VGCCs, increasing local $Ca^{2+}$ concentration to levels as high as 100μM to trigger neurotransmitter release (Augustine, 2001; Hsu et al., 1996; Heidelberger et al., 1994). This low affinity of $Ca^{2+}$ for the exocytotic trigger, together with the localisation of VGCCs at the active zone, produces a high fidelity and speed of release of neurotransmitter to occur within 1 ms of $Ca^{2+}$ entry into the nerve terminal (Bruns and Jahn, 1995). Given the background of the contribution of $Ca^{2+}$ to exocytosis, one could postulate a mechanism of modulating presynaptic function by altering the coupling of $Ca^{2+}$ to neurotransmitter release. Indeed, studies have shown that both protein kinase A (PKA) and protein kinase C (PKC) can phosphorylate $Ca^{2+}$ channels to facilitate their opening, thereby affecting neurotransmission (Dolphin, 1995; Farago and Nishizuka, 1990). In addition, $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) has been proposed to affect glutamate release by potentially phosphorylating VGCCs to modulate $Ca^{2+}$ entry in synaptosomes, although this has not been directly shown (Sihra, 1993).

### 1.2.3. The exocytotic machinery

The third target at which modulation of presynaptic function modulation can be exerted is at the release machinery itself. A number of proteins known as SNAREs

(soluble N-ethylmaleimide sensitive factor [NSF]-attachment protein receptors) found on the SVs (designated v-SNAREs) or in the target plasma membrane (designated t-SNAREs) have been associated with the regulation of neurotransmitter release (Murthy and De Camilli, 2003; Sollner, 2003; Sollner et al., 1993). These include two t-SNAREs, syntaxin, a 35kDa protein shown to associate with VGCCs and SNAP-25, a 25kDa protein; and a 19kDa v-SNARE synaptobrevin or VAMP (vesicle-associated membrane protein).

During exocytosis, SVs are first docked onto the plasma membrane at the active zone. They then undergo priming in preparation for fusion with the membrane and release their contents, after which SVs are recycled by ways of endocytosis. The docking process is initiated when nSec1, also known as Munc 18, dissociates from syntaxin (v-SNARE) via a mechanism involving active zone-specific proteins Munc 13 (mammalian homologue of *Caenorhabditis elegans* unc 13) and RIM 1 (a Rab3-interacting molecule-1) (Rizo and Sudhof, 2002; Pevsner et al., 1994). Together, these proteins facilitate a change of conformation state of syntaxin from a 'closed' state (bound to nSec1) to an 'open' state, enabling it to bind to SNAP-25 to form a binary SNARE complex (Fasshauer, 2003). A stable quaternary SNARE complex is then formed when the v-SNARE synaptobrevin binds and, in so doing, brings the membranes of SV and that of the active zone in close apposition with each other (Fasshauer, 2003; Benfenati et al., 1999; Lin and Scheller, 1997). Studies have shown that disruption of this quaternary SNARE complex using botulinum neurotoxins causes severe blockade of exocytosis (Bajohrs et al., 2004).

Once docked, the SNARE complex undergoes ATP-dependent priming where the ATP-hydrolysing protein NSF (NEM-sensitive factor) and SNAPs (NSF attachment protein) may be involved, although this is still under debate ((Lin and Scheller, 2000; Mayer et al., 1996; Sollner et al., 1993), see later). In addition, the protein Munc 13 is believed to play a role in vesicle priming where acquisition of fusion competence

comes from lipid and protein changes (Murthy and De Camilli, 2003; Rhee et al., 2002).

Next, SVs are fused with the plasma membrane. The mechanism of this fusion process is still under considerable debate. Whilst some models suggest that ATP hydrolysis of NSF may act to facilitate fusion, others propose that it is the disassembly, not the NSF-mediated assembly of the SNARE complex that drives fusion (Jahn and Sudhof, 1994). However, as no recruitment of SNAPs/NSF has been shown before or after fusion (Banerjee et al., 1996; Mayer et al., 1996), the precise role of SNAPs/NSF in fusion remains to be elucidated.

Increasingly, studies have suggested the $Ca^{2+}$ triggering of fusion is mediated through a 65kDa vesicular protein, synaptotagmin, which is believed to act as a $Ca^{2+}$ sensor to trigger release in response to $Ca^{2+}$ influx. Indeed, studies have shown that synaptotagmin plays a role in synaptic transmission where synaptotagmin knockout in C. elegans displays severe defects in locomotion, feeding and defecation (Nonet et al., 1993). Similarly, the mouse knockout also displayed a decreased amount of fast-evoked $Ca^{2+}$-dependent neurotransmitter release (Geppert et al., 1994) and a slower vesicle fusion (Nicholson-Tomishima and Ryan, 2004). Synaptotagmin has been shown to have binding sites for $Ca^{2+}$, syntaxin (Kee and Scheller, 1996), SNAP-25 (Schiavo et al., 1997) as well as for phospholipids in the membrane bilayers (Li et al., 1995a). Thus, the fusion process may involve the interaction between synaptotagmin, syntaxin and $Ca^{2+}$, where it is dependent on syntaxin to regulate $Ca^{2+}$ entry (Zamponi, 2003; Stanley, 2003) and synaptotagmin to sense changes in local $[Ca^{2+}]_i$ (Augustine, 2001) to effect efficient fusion. The resulting binding of $Ca^{2+}$ causes synaptotagmin to change its conformation and induces "zippering" of the parallel helices in the SNARE complex to enhance fusion for neurotransmitter release. Furthermore, there are suggestions that fusion may involve the formation of a putative proteinacious fusion pore with a 38kDa channel-forming protein, synaptophysin (Knaus et al., 1990). This

formation of fusion pore may be assisted by complexin (also known as synaphin) (Tokumaru et al., 2001), a cytosolic protein which acts to promote SNARE interaction, and thereby enables their oligomerisation through a mechanism that is yet to be determined.

As can be seen, control of exocytosis is a highly regulatable process, involving the recruitment and formations of complexes between SNAREs and interacting proteins to mediate a fine presynaptic response. Thus, the availability and subcellular localisation of proteins as well as factors that affect their activation or interactions are important in regulating the efficacy of release. For example, the SNAREs and their interacting proteins RIM1 and Munc13 are all found enriched in the presynaptic active zone (Sollner, 2003; Murthy and De Camilli, 2003; Calakos et al., 2004; Betz et al., 2001). In addition, release could be modulated by altering levels of proteins such as RIM, Munc 13, rabphilin and synaptotagmin, all of which have C2 domains sensitive to the binding of $Ca^{2+}$. Studies have shown that reducing levels of these proteins may result in a reduction in the cooperative relation between $Ca^{2+}$ concentration and exocytosis (Stewart et al., 2000). Furthermore, both syntaxin and SNAP-25 have been shown to be phosphorylated by PKA, leading to an increased interaction with synaptotagmin and an enhancement in SV release (Nagy et al., 2004; Hepp et al., 2002; Risinger and Bennett, 1999). The phosphorylation of synaptotagmin by CaMKII has been shown to be necessary for the maximal binding of syntaxin to synaptotagmin in synaptosomal preparations (Verona et al., 2000). NSF has also been shown to undergo a depolarisation-induced and $Ca^{2+}$-dependent phosphorylation by PKC to result in the inhibition of fusion (Matveeva et al., 2001).

### 1.2.4. Regulation of synaptic vesicle trafficking via synapsin

Control of presynaptic function can be exerted upstream of SV exocytosis to modulate the trafficking of SVs to the active zone. In brief, SVs in the nerve terminal are organised into two functionally distinct synaptic pools. The first subpopulation of

SVs, the readily releasable pool (RRP), are primed and release-competent vesicles found docked onto the plasma membrane at the active zone (Schikorski and Stevens, 2001). The reserve pool (RP) represents the second pool of SVs that are not morphologically docked or primed for fusion, and is more distal from the active zone and constitutes a source for replenishing the releasable pool during activity-dependent depletion (Benfenati et al., 1999; Neher, 1998; Greengard et al., 1993). SVs of this RP are found associated with actin filaments through the synaptic protein, synapsins (Fon and Edwards, 2001; Sudhof, 2000; Betz and Angleson, 1998). Upon synaptic stimulation, a cycle of exocytosis and endocytosis takes place (known as the SV cycle) where SVs from the RP are translocated to the active zone to dock onto the plasma membrane at the active site to replenish the RRP for exocytosis.

After exocytosis, the emptied vesicle is then retrieved back to the cytosol via clathrin-coat dependent (Takei and Haucke, 2001; Maycox et al., 1992) or independent (Schmidt et al., 1999) endocytosis and recycled to be refilled with new neurotransmitters. In some cases, there is evidence that a fast 'kiss and run' exocytosis takes place with successive rounds of transient vesicle fusion and the incomplete collapse of vesicle membranes (Gandhi and Stevens, 2003; Aravanis et al., 2003; Stevens and Williams, 2000). This has been suggested with experiments showing the lack of styryl dye FM1-43 destaining, which normally labels the inside leaf of the vesicle membrane (Henkel and Almers, 1996). In addition, the lack of staining with antibodies to luminal synaptophysin epitopes after intense stimulation suggests that the synaptophysin-containing SV membrane was reinternalised quickly (Valtorta et al., 1988).

In general, the entry of SVs into RRP is considered as an important rate-limiting step for the control of neurotransmitter release. Studies have shown that the ability of a nerve terminal to release neurotransmitters not only depends on the size of RRP and the recycling capacity, but also greatly on the sizes of RP and the rate of mobilisation

of SVs from this RP to the releasable pool (Benfenati et al., 1999). In the latter case, SV mobilisation is mediated by a series of specific interactions between the cytoskeleton, SVs, presynaptic membrane and cytosolic proteins that act together to provide fine regulation of the exocytotic machinery. These interactions, in turn, are regulated by a number of proteins and mediators that are found localised in the active zone. One such protein, synapsin I, a SV tethering protein has been of particular interest in the control of SV availability.

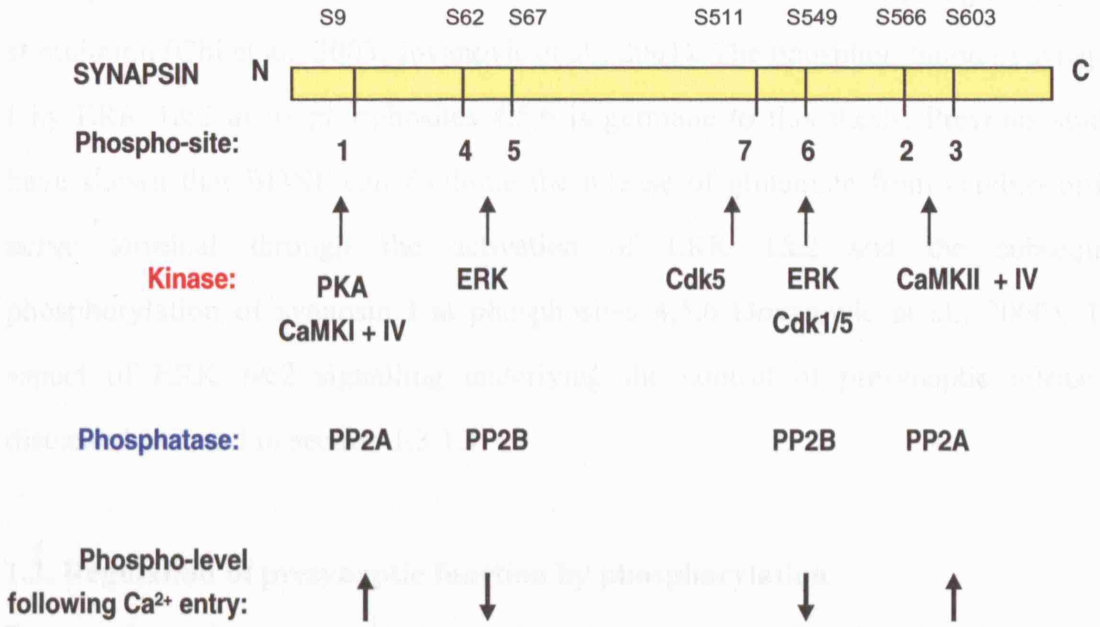
Synapsin I is a member of the synapsin family found associated with the cytoplasmic surface of the SV membrane (Greengard et al., 1993; De Camilli et al., 1990). Upon $Ca^{2+}$ entry, it is one of the most prominent phosphoproteins that is regulated in response to changes in the cytosolic $Ca^{2+}$ levels. Synapsin I has been shown to tether SVs to the actin cytoskeleton (Hilfiker et al., 1999; Greengard et al., 1993; De Camilli et al., 1990) as well as cross-linking adjacent SVs to form SV clusters (Benfenati et al., 1992a) and, in so doing, regulates the proportion of vesicles in the nerve terminal available for release (Benfenati et al., 1999). These functions of synapsin I have been confirmed in experiments with the synapsin I deficient mice where a markedly decreased release of glutamate and abnormal synaptic transmission were observed (Li et al., 1995b). In addition, depletion of synapsin has been shown to result in reduction in the number of SVs in the nerve terminal, depletion of SVs in the RP, as well as alterations in the synaptic plasticity phenomena (Benfenati et al., 1999; Hilfiker et al., 1999; Ryan et al., 1996; Pieribone et al., 1995).

The binding ability of synapsins to SVs and to actin is controlled in a phosphorylation-dependent manner. Studies have revealed that phosphorylation of synapsin I promotes its translocation from particulate (binding to vesicle and cytoskeleton) to cytosolic soluble fraction (cytosol), consistent with what would be predicted following depolarisation of the nerve terminal and $Ca^{2+}$-dependent activation of phosphorylation (Sihra et al., 1989). Experiments using green

fluorescent protein-labelled synapsin Ia in hippocampal cell cultures have also demonstrated that the dispersion of synapsin I occurs before RP pool mobilisation and exocytosis, and that this is controlled tightly by phosphorylation and dephosphorylation of synapsin I in response to different neuronal stimulation frequencies (Chi et al., 2003; Chi et al., 2001). Thus, under resting condition, synapsins tether the SVs to the actin filaments present in the presynaptic sites. During synaptic activity, synapsins are phosphorylated, dissociate from the vesicles and thereby allow entry of SVs into the RRP at the active zone. Once stimulation is terminated, depending on the overall phosphorylation state of synapsin (see below), the protein reassociates with SVs and with the cytoskeleton.

The phosphorylation state of synapsin is mediated by several protein kinases and phosphatases at seven specific phosphorylation sites **(Schematic 1.2.4)** (Jovanovic et al., 2001): site 1 (Ser 9) is phosphorylated by both cAMP-dependent protein kinase (PKA) and $Ca^{2+}$/calmodulin-dependent protein kinase I and IV (CaMKI and IV) and dephoshorylated by protein phosphatase 2A (PP2A); site 2/3 (Ser 566, 603) is phosphorylated by CaMKII and IV and is also a target for PP2A; site 4/5 (Ser 62, 67) is phosphorylated by extracellular signal-regulated kinase (ERK) and dephosphorylated by protein phosphatase 2B (PP2B, also known as calcineurin); site 6 (Ser 549) is phosphorylated by ERK and cyclin-dependent kinase 1 and 5 (cdk1 and 5) and dephosphorylated by PP2B respectively; site 7 (Ser 511) is phosphorylated by cdk5.

The function of synapsin in controlling the availability of vesicles for release is, therefore, mediated by opposing effects of increasing phosphorylation of synapsin phosphosites 1,2,3 and 4,5,6 and decreasing phosphorylation at sites 4,5,6 (Jovanovic et al., 2001). Studies have reported that the degree of stimulation or depolarisation is important in regulating these opposing effects (Chi et al., 2003). Whilst phosphorylation of synapsin at the CaMK phosphosite 1,2,3 and ERK phosphosite




**Schematic 1.2.4. Protein kinases and phosphatases acting on synapsin I:** PKA, cAMP-dependent protein kinase; CaMKI, CaMKII and CaMKIV, $Ca^{2+}$/calmodulin dependent protein kinases I, II and IV; ERK, extracellular signal-regulated protein kinase; cdk1/5, cyclin-dependent protein kinase 1 and 5; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B/calcineurin. Following $Ca^{2+}$ entry, synapsin is phosphorylated on sites 1,2,3 by CaMKI, CaMKII and PKA and dephosphorylated at site 4,5,6 by calcineurin.

4,5,6 enhances release by freeing the vesicles to the RRP site at low frequency stimulation, dephosphorylation at phosphosites 4,5,6 by $Ca^{2+}$-dependent activation of calcineurin limits the ability of ERK phosphorylation impinging on the synapsin, and thereby provides a constraint to neurotransmitter release during high frequency stimulation (Chi et al., 2003; Jovanovic et al., 2001). The phosphorylation of synapsin I by ERK 1&2 at its phosphosites 4,5,6 is germane to this thesis. Previous studies have shown that BDNF can facilitate the release of glutamate from cerebrocortical nerve terminal through the activation of ERK 1&2 and the subsequent phosphorylation of synapsin I at phosphosites 4,5,6 (Jovanovic et al., 2000). This aspect of ERK 1&2 signalling underlying the control of presynaptic release is discussed in detail in section 1.3.1.1.

## 1.3. Regulation of presynaptic function by phosphorylation

From the above description, the importance of phosphorylation and dephosphorylation in regulating the function of synapsin I underlying the control of neurotransmitter release is self-evident. This is also true for many presynaptic mechanisms which involve the activation of many presynaptic kinases and phosphatases. In the following section, the activation and impact of these kinases and phosphatases particularly in presynaptic functions are discussed.

### 1.3.1. Protein kinases

In eukaryotes, there are over 1000 protein kinases involved in mediating phosphorylation of substrate proteins. These kinases operate by transferring negatively charged phosphate group from ATP to the serine, threonine or tyrosine residues on the substrate (Engh and Bossemeyer, 2001) which subsequently leads to alteration in the behaviour of the protein. The precise activation of these protein kinases, therefore, becomes an important means by which activities of signalling proteins in the cell can be regulated following activity-dependent stimulation. Several protein kinases found presynaptically, such as PKA, PKC, CaMKII, ERK 1&2, PI3K

and cdk5, have been shown to be activated in response to changes in the levels of second messengers such as $Ca^{2+}$, cyclic-AMP and phospholipids. Their activation, in turn, contributes to the regulation of downstream signalling in a highly specific manner. The focus of this thesis is on the activation of ERK 1&2, CaMKII, PI3K and cdk5 and their cross-talk in the modulation of presynaptic function. Thus, these kinases will be described further in the following section.

#### 1.3.1.1. Extracellular signal-regulated kinase 1&2 (ERK 1&2)

Extracellular signal-regulated protein kinases 1&2 (ERK 1&2) belong to the mitogen-activated protein kinase (MAPK) superfamily that represents a family of proline-directed serine/threonine protein kinases. These MAPKs mediate the phosphorylation of serine or threonine residues directly upstream of proline. Apart from ERK 1&2, the two other major subfamilies of MAPK include: c-Jun N-terminal kinases (JNK 1, 2 & 3) and p38 proteins, all of which consist of a homodimer of a 80kDa subunit and contain the signature sequence -TXY-, where T and Y are threonine and tyrosine and X is glutamate, proline or glycine, in ERK, JNK and p38 respectively (Pearson et al., 2001). MAPKs are activated by dual phosphorylation of both threonine and tyrosine within this signature sequence by the action of upstream MAPK kinases such as MAPK kinase 1&2 or MEK 1&2. Each subfamily of MAPKs can be activated by different stimuli. ERK 1&2 are generally activated in response to neurotrophic factors or cell survival signals. In contrast, JNK and p38 kinases are stress-activated protein kinases (SAPKs) which have been shown to be particularly associated with stress and inflammatory stimuli (Young et al., 1997).

ERK 1 and 2, the two isoforms of ERK (p44 and p42), are the focus of this thesis. Structural analysis of ERK 1&2 have indicated that they are composed of a large C-terminal domain and a small N-terminal domain linked together by a crossover region, with the catalytic domain found at the interface of the two domains (Cobb and Goldsmith, 1995). Activation of ERK 1&2 occurs when upstream MEK 1&2

phosphorylate both the Thr183 and Tyr185 (on rat ERK) or Thr202 and Tyr204 residues (on human ERK) and, in so doing, change the conformation state of the kinase to expose previously inaccessible active sites to downstream substrates (Payne et al., 1991).

The function of ERK 1&2 has been extensively studied over the years with implications of this cascade in the control of many forms of synaptic plasticity (Thomas and Huganir, 2004; Sweatt, 2004). These include LTP in the hippocampus (English and Sweatt, 1997), dentate gyrus (Coogan et al., 1999), cortex (Di Cristo et al., 2001) and amygdala (Huang et al., 2000). During LTP, ERK 1&2 activation has been shown to be necessary for the CaMKII-mediated increase in AMPA-R transmission, phosphorylation of transcription factors (Wu et al., 2001a; Vanhoutte et al., 1999), as well as regulating the function of voltage-dependent $K^+$ channels (Adams et al., 2000). Interestingly, inhibition of ERK signalling has been shown to prevent LTD in cultured Purkinje cells by activating metabotropic glutamate receptors (mGluR) which are required for LTD to occur (Kawasaki et al., 1999). However, despite the fact that ERK 1&2 are clearly involved in the regulation of synaptic plasticity, the degree of control by which presynaptic activation of ERK 1&2 contributes to this plasticity is not known.

Recently, studies have suggested that induction of LTP and/or LTD is reflected by changes in the secretion of endogenous neurotrophin BDNF (brain-derived neurotrophic factor) (Aicardi et al., 2004). Consistent with this, work in dentate gyrus synaptosomes has revealed that the BDNF-induced LTP is accompanied by the activation of presynaptic TrkB receptors, activation of ERK 1&2 and the increase of glutamate release (Gooney et al., 2004; Gooney et al., 2002). This suggests a mechanism by which postsynaptically-released BDNF may serve as a retrograde messenger during activity-dependent conditions to modulate synaptic plasticity by activating neurotransmitter release via the activation of presynaptic ERK 1&2.

Indeed, a growing body of evidence suggests that ERK 1&2 are prominent targets of BDNF-mediated signalling (Poo, 2001) and that their activation effects the facilitation of glutamate release (Chi et al., 2003; Jovanovic et al., 2000; Chin et al., 2002). Studies have shown that BDNF can facilitate presynaptic glutamate release from rat cerebrocortical synaptosomes where this enhancement of release was sensitive to the MEK inhibitor PD98059 as well as being severely attenuated in synapsin-deficient mice (Jovanovic et al., 2000). This suggests that activation of ERK 1&2 by BDNF leads to an increase in synapsin phosphorylation to subsequently cause the enhancement in neurotransmitter release (Jovanovic et al., 2000). The role of ERK-dependent phosphorylation of synapsin in the control of exocytosis has also been highlighted in studies where synapsin dynamics and SV pool turnover was shown to be affected by synapsin phosphorylation at phosphosites 4,5,6 following stimulation of the nerve terminal (Chi et al., 2003; Chin et al., 2002). Unlike CaMKII, however, the modulation by ERK 1&2 appears to modulate just actin dynamics rather than the binding affinity of synapsin to SVs and actin (Jovanovic et al., 1996).

Taken together, the above evidence suggests an involvement of presynaptic ERK 1&2 activation in the modulation of a key synaptic function such as neurotransmitter release. The fact that activation of ERK 1&2 occurs following neurotrophin (Chin et al., 2002; Jovanovic et al., 2000) and electrical stimulation (Chi et al., 2003) suggests that ERK 1&2 are activity-dependent molecules that serve to provide presynaptic regulation. This thesis is concerned with the elucidation of the molecular signalling pathways leading to ERK 1&2 activation in the presynaptic nerve terminal. Much of the work will be focused on examining the potential activators as well as regulators and/or mediators that are involved in the ERK 1&2 signalling, including activation in response to $Ca^{2+}$ and neurotrophic factors.

#### 1.3.1.2. $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII)

$Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) is a multifunctional serine/threonine

kinase made up of 6 to 12 subunits, primarily the 52kDa α isoform and 60kDa β isoform (Miller and Kennedy, 1985). Subunits are held together by association domains in the C-terminals, which form a central globular structure from which the N-termini extend radially (Kanaseki et al., 1991). The catalytic site and the autoinhibitory domain are found at the N-terminus and, during basal conditions, these sites are bound to each other to render CaMKII inactive. CaMKII is activated by the binding of $Ca^{2+}$ and calmodulin (CaM), which causes a conformational change to relieve autoinhibition and expose the enzyme to be autophosphorylated at Thr286 in the α subunit and Thr287 in the β subunit. This causes CaMKII to remain activated even when $Ca^{2+}$ returns to sub-stimulation levels (Ninan and Arancio, 2004; Lai et al., 1986; Miller and Kennedy, 1986). This unique configuration of CaMKII enables the enzyme to mediate an effective downstream $Ca^{2+}$-dependent signalling such that a transient $Ca^{2+}$ signal can be converted to a signal that outlasts the duration of the original $Ca^{2+}$ pulse (Lisman et al., 2002; Thiagarajan et al., 2002; Lai et al., 1986; Miller and Kennedy, 1986). CaMKII is commonly found as a heteromers, although homomers are also possible (Brocke et al., 1999; Kanaseki et al., 1991). There is evidence that αCaMKII is more selective for higher levels of $Ca^{2+}$ while βCaMKII is better at sensing lower $Ca^{2+}$ levels (Thiagarajan et al., 2002; Brocke et al., 1999). Thus, as heteromers, they are capable of tuning neuronal responses to different levels of activities.

The ability of CaMKII to phosphorylate synapsin seems to be key to modulating presynaptic neurotransmitter release. Early studies in squid giant synapse and rat brain synaptosomes indicated that CaMKII has a role in increasing depolarisation-induced neurotransmitter release including glutamate and noradrenaline (Llinas et al., 1991; Lin et al., 1990; Nichols et al., 1990; Sihra et al., 1989), where this CaMKII-mediated facilitation of release is accompanied by an increased phosphorylation of synapsin at phosphosites 2,3. Conversely, this effect of release potentiation is attenuated with the addition of a CaMKII inhibitory peptide. Consistent with this, the rate of synapsin I

dispersion and SV pool turnover is controlled by $Ca^{2+}$-dependent phosphorylation of the CaMK sites of synapsin 1a (Chi et al., 2003; Sihra et al., 1989).

### 1.3.1.3. Phosphoinositol-3-kinase (PI3K)

The class I phosphoinositol-3-kinase (PI3K) is a family of lipid kinases that catalyse the conversion of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) to the lipid product phosphatidylinositol-3,4,5-triphosphate ($PIP_3$). There are two subclasses of class I PI3K, class Ia and Ib, which differ in their subunit composition and the mechanism of activation. Class Ia PI3K, including the catalytic subunit p110α, 110β or p110δ which associates with the regulatory p85 subunit, are activated following stimulation of RTKs such as neurotrophin receptors or growth receptors (Yuen and Mobley, 1999; Soltoff et al., 1992). This class of PI3K has also been shown to be activated directly by Ras that can interact with PI3K through the p110 subunit (Downward, 1998; Rodriguez-Viciana et al., 1994). Class Ib PI3K, on the other hand, consists of p110γ subunit which complexes with a regulatory p101 subunit (Vanhaesebroeck and Waterfield, 1999). These class Ib PI3Ks are activated by heterotrimeric G proteins upon GPCR stimulation.

The production of $PIP_3$ by PI3K enables a variety of downstream lipid-mediated signalling. One key downstream target of PI3K and $PIP_3$ is Akt or PKB (protein kinase B), a PH-domain containing serine/threonine protein kinase (Kaplan and Miller, 1997; Franke et al., 1997; Burgering and Coffer, 1995). Activation of Akt has been shown to promote cell survival by phosphorylating substrates such as inhibitory pro-apoptotic factors and transcription factors (Kane et al., 1999; Cardone et al., 1998). In addition, PI3K can activate effectors such as GTPase Rac to mediate downstream pathways involved with cytoskeletal reorganisation (Posern et al., 2000).

Studies have shown that the PI3K-mediated signalling pathway is important in

cellular functions such as cell survival and cell differentiation in both non-neuronal and neuronal systems (Stephens et al., 2005; Katso et al., 2001; Vanhaesebroeck and Waterfield, 1999; Gottschalk et al., 1999). In neuroendocrine cells, PI3K have been demonstrated to be involved in regulating the release of secretory granules where impairment or inactivation of PI3K results in the blockade of the ATP-dependent priming of exocytosis (Meunier et al., 2005). In PC12 cells, overexpression of PI3K has been shown to induce neurite outgrowth and is important for the maintenance of neuronal morphology (Sanchez et al., 2004). In addition, PI3K has been shown to be involved in the regulation of plasticity by modulating BDNF-potentiation of synaptic output and dopamine release from slices and synaptosomes of the corpus striatum (Goggi et al., 2003). The involvement of PI3K in the regulation of release has also been supported by findings that PI3K is implicated in the delivery of SVs from the RP to replenish the RRP (Cousin et al., 2003). Taken together, this suggests PI3K as an important regulator for molecular signalling underlying presynaptic modulation.

#### 1.3.1.4. Cyclin-dependent kinase 5 (cdk5)

Cyclin-dependent kinase 5 (Cdk5), also known as Cdc2-like kinase, is a proline-directed serine/threonine kinase that belongs to the cyclin-dependent kinase family (cdks). Cdks was originally found in human cell lines as postmitotic regulators that are involved in controlling cell differentiation in neuronal and muscle cells. However, unlike the majority of cdks, cdk5 does not participate in cell cycle regulation and is not activated by cyclin (Xiong et al., 1992). Instead, cdk5 kinase activity is largely detected in the nervous systems and has been implicated in neuronal functions such as neurite outgrowth (Nikolic et al., 1996), neuronal migration (Smith et al., 2001), modulation of neuronal cytoskeleton structure and dynamics (Smith et al., 2001; Dhavan and Tsai, 2001), exocytosis (Tomizawa et al., 2002; Fletcher et al., 1999) and endocytosis (Lee et al., 2004; Tomizawa et al., 2003; Tan et al., 2003). In humans, dysregulation of cdk5 has been shown to be linked with neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Alvira et al., 2006; Giese et al., 2005).

At the cellular level, cdk5 is activated by the binding of cyclin-related protein activators, p35 and p39, which are present at the synaptic membrane (Ko et al., 2001). These activators act to bind to the catalytic cleft of cdk5 and interact with both the N- and C-terminal lobes to form a large protein complex. On binding, they trigger phosphorylation at Ser 159, so that cdk5 is activated to phosphorylate its substrates. p35 is rapidly cleaved into a more stable protein of 25kDa (p25) by the $Ca^{2+}$-dependent activation of calpain (Hisanaga and Saito, 2003; Lee et al., 2000b; Tsai et al., 1994). Thus, p25 is released into the cytosol along with the active form of cdk5 to prolong the activation of cdk5 for the mediation of signalling downstream.

The functions of cdk5 are still being elucidated. Over the years, there have been debates over the potential involvement of cdk5 as a regulator of neuronal survival or neuronal death. Whereas studies on cdk5 knockout mice revealed a role of cdk5 in neuronal survival (Tanaka et al., 2001), other reports appear to suggest a contradicting role of cdk5 in regulating neuronal death pathways where cdk5 potentiates the activation of JNK (Otth et al., 2003). A recent report suggested that cdk5 might influence neuronal survival by downregulating the activity of ERK 1&2 by phosphorylating MEK 1&2 (Sharma et al., 2002). In addition, cdk5 have been shown to be capable of phosphorylating proteins including neurofilament proteins, actin-binding proteins, proteins of the SVs, as well as tau and MAP1b (Weishaupt et al., 2003). Presynaptically, cdk5 has been shown to phosphorylate synapsin on site 6 (Jovanovic et al., 1996), although the mechanism and impact of this modulation remains to be established.

### 1.3.2. Protein phosphatases

There are three major classes of serine/threonine protein phosphatases, termed protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B also known as calcineurin), all of which are found in the brain. These phosphatases effect the removal of phosphate groups from their substrates, and

thereby, result in the alteration of the protein's function. Presynaptically, PP2A and calcineurin have been shown to dephosphorylate synapsin at their phosphosites 1, 2/3 and 4,5,6 respectively, to influence delivery of SVs to the RRP (Jovanovic et al., 1996). A recent report has suggested that PP2A and calcineurin exert their effects differentially on the delivery of SVs to the membrane (Baldwin et al., 2003). It is proposed that while PP2A influences only the fully fusion process, PP2B primarily influences the kiss-and-run mode. However, the exact mechanism of this regulation and its relevance requires confirmation.

### 1.3.2.1. Protein phosphatase 1 (PP1)

Protein phosphatase 1 (PP1) is widely expressed in the brain (Strack et al., 1999; Allen et al., 1997) where its α, β, γ1 and γ2 isoforms are differentially distributed. Pharmacologically, PP1, along with PP2A, is inhibited by the cell-permeant toxins okadaic acid and calyculin A, as well as by cell-impermeant microcystin (Winder and Sweatt, 2001). The regulation of PP1 activity is a complex mechanism, involving proteins such as inhibitor-1 (Sakagami et al., 1994) and DARPP-32 (Walaas et al., 1983), both of which are phosphorylation targets of PKA. Thus, phosphorylation of inhibitor-1 or DARPP-32 by PKA promotes the binding of these proteins to PP1, allowing them to mediate inhibition of PP1 activity. This inhibition of PP1 could be relieved by the dephosphorylation of the inhibitor-1 or DARPP-32 PKA phospho-site by calcineurin (Winder and Sweatt, 2001). In addition, PKA can have a positive regulatory role in controlling PP1 activity. Studies have shown that PKA can phosphorylate an actin-binding protein called neurabin, and in so doing, reduce the neurabin-mediated inhibition of PP1 (McAvoy et al., 1999). Apart from indirect modulation by PKA phosphorylation, the activity of PP1 can also be controlled through subcellular compartmentalisation. In neurons, the binding of phosphatase with sphinophilin and/or neurabin can inhibit PP1 as well as target PP1 to actin and postsynaptic densities, thereby presenting the phosphatase for interaction with various membrane receptors (Winder and Sweatt, 2001; Smith et al., 1999; Allen et al., 1997).

PP1 has been shown to have various functions including the reversal of LTP and the induction of LTD. Studies have revealed that PP1 is capable of dephosphorylating the membrane-bound form of CaMKIIα (Strack et al., 1997a), which is normally autophosphorylated during LTP induction. In addition, dephosphorylation of GluR1 (Lee et al., 2000a) and CaMKIIα in the post-synaptic density by PP1 contributes to the synaptic induction of LTD (Winder and Sweatt, 2001). Thus, the ability of excitatory synapses to undergo synaptic plasticity may depend on the synaptic input and the regulation of induction by PP1, which functions to change the properties of important proteins involved in generating this synaptic plasticity.

### 1.3.2.2. Protein phosphatase 2A (PP2A)

Protein phosphatase 2A (PP2A) is a heterotrimeric holoenzyme consisting of 36kDa catalytic subunit (C), 65kDa structural subunit (A) and the 55kDa regulatory subunit (B). Whereas subunits A and C are broadly expressed, B subunits are enriched in the brain (Winder and Sweatt, 2001). The activity of PP2A can be regulated differentially depending on its subunit composition, as well as modulation by phosphorylation. Phosphorylation of the Thr304 and Tyr307 residues in the C subunit by protein kinases could result in the inactivation of PP2A, which could otherwise be reversed by the ability of PP2A to undergo auto-dephosphorylation (Brautigan, 1995). Dephosphorylation of proteins by PP2A has been implicated in the regulation of a number of signalling cascades. Studies have shown that PP2A can positively regulate Ras signalling by dephosphorylating kinase suppressor of Ras (KSR) and Raf-1 (Dougherty et al., 2005; Ory et al., 2003). However, the effects of PP2A appear to be bi-directional as there is evidence that PP2A also has an inhibitory effect on protein kinases such as Akt/PKB, PKC and ERK (Van Kanegan et al., 2005; Andjelkovic et al., 1996; Keranen et al., 1995) by directly dephosphorylating these kinases. Examination of PP2A in cultured neurons also showed an inhibition of mGluR-mediated ERK 1&2 activation (Mao et al., 2005). This negative regulation of synaptic

function is further supported by postsynaptic studies which suggest a role of PP2A in the induction of LTD by clustering of AMPA receptors at synapses between granule cells and Purkinje cells (Launey et al., 2004), as well as regulating the dephosphorylation of GluR1 and soluble CaMKIIα (Winder and Sweatt, 2001). Presynaptically, potentiation of depolarisation-induced neurotransmitter release has been demonstrated using okadaic acid which, taken together with *in vitro* analysis, indicates PP2A as the principle phosphatase that dephosphorylates synapsin at site 1 and 2,3 (Jovanovic et al., 2001). In addition, PP2A may also regulate exocytosis by modulating at the level of $Ca^{2+}$ entry as it has been shown to promote dephosphorylation of VGCCs (Hall et al., 2006; Davare et al., 2000).

### 1.3.2.3. Protein phosphatase 2B (PP2B)

Protein phosphatase 2B or calcineurin is a $Ca^{2+}$/calmodulin-dependent Ser/Thr phosphatase that is found enriched in neuronal tissue (Jiang et al., 1997; Steiner et al., 1992) and is rapidly activated upon $Ca^{2+}$ entry (Winder and Sweatt, 2001). Pharmacologically, it can be distinguished from PP1 and PP2A. Calcineurin is relatively insensitive to inhibitors of PP1 and PP2A, but is inhibited by the immunosuppressive compounds such as FK506 and cyclosporin A, via an immunophilin intermediary (FK506-binding protein or cyclophilin, respectively) (Winder and Sweatt, 2001). Calcineurin consists of a catalytic A subunit (60kDa) and a myristoylated B regulatory subunit (19kDa), where the A subunit binds one $Ca^{2+}$/CaM complex and the B subunit is structurally related to CaM. The activity of calcineurin is regulated by $Ca^{2+}$ and calmodulin in a highly cooperative manner. The phosphatase activity further increases when $Ca^{2+}$ binds to the B-subunit. This cooperativity is explained by the identification of an autoinhibitory domain in A-subunit (residues 467-492), such that the binding of $Ca^{2+}$/CaM is believed to disrupt the interaction of the CaM binding domain and B-subunit to induce a conformational change and relieve the catalytic domain from autoinhibition (Griffith et al., 1995).

Besides having postsynaptic functions regulating glutamate receptor channels such as NMDA receptors and supporting long-term depression (LTD) (Winder and Sweatt, 2001), calcineurin has been reported to suppress exocytotic release of glutamate from rat cortical synaptosomes (Sihra et al., 1995). Thus, the increase in basal and depolarisation-evoked $Ca^{2+}$ levels during calcineurin inhibition (Sihra et al., 1995) suggests that calcineurin exerts its modulation at the level of $Ca^{2+}$ entry. Indeed, there is evidence that calcineurin is capable of dephosphorylating the β-subunit of L-type VGCC as well as on PKA-dependent phosphosites in the voltage-gated sodium channels (Yakel, 1997). In conjunction with this, analysis of high-voltage-activated $Ca^{2+}$ currents also support the role of calcineurin in the negative feedback regulation of $Ca^{2+}$ entry (Burley and Sihra, 2000). Furthermore, cyclosporin A showed an acceleration in exocytosis from synaptosomes induced by ionomycin (Jovanovic et al., 2001), indicating the action of calcineurin exerted downstream of $Ca^{2+}$ influx through the dephosphorylation of synapsin phosphosites 4,5,6. Thus, these data indicate that the $Ca^{2+}$-dependent activation of calcineurin plays an important modulatory role in synaptic plasticity, both postsynaptically and presynaptically.

### 1.3.3. Receptor tyrosine kinases (RTKs)

Receptor tyrosine kinases (RTKs) are a class of cell-surface receptors for growth factors and other extracellular signals which have been implicated in a number of functions including cell survival, cell differentiation, migration, as well as apoptosis (Huang and Reichardt, 2003; Poo, 2001; Kaplan and Miller, 2000). Once bound by their ligand, RTKs dimerise and become catalytically active to mediate autophosphorylation on tyrosine residues-containing motifs, thereby generating recognition sites for the recruitment of substrates and/or adaptor proteins to the plasma membrane for further downstream signalling. The activities of RTKs are negatively controlled by protein tyrosine phosphatases (PTPs), which mediate dephosphorylation of ligand-activated RTKs (Ostman and Bohmer, 2001; Fiorini et al., 2001).

Neurotrophins are a highly homologous family of protein growth factors, consisting of four main members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4). Neurotrophins act by binding to RTKs called Trks (tropomysin-related kinases). Three Trk receptors have been discovered to-date, namely TrkA, TrkB and TrkC, all of which are high affinity receptors (Kd ~ $10^{-11}$M) that bind to members of the neurotrophin family with differential affinities (Huang and Reichardt, 2003; Poo, 2001). For example, TrkA is specific for NGF and TrkB for BDNF and NT-3. Apart from Trks, all members of the neurotrophins are capable of binding to a low affinity receptor (Kd ~ $10^{-9}$M) known as $p75^{NTR}$. P75 belongs to the TNF (tumour necrosis factor) receptor family and can interact with the Trk receptors to mediate neurotrophic effects. The responsiveness of the Trk receptor to the neurotrophin depends also on whether or not Trk receptors are expressed on the surface of the cell. In most cases, the insertion of Trk into the cell surface can be determined by electrical activity, cAMP and $Ca^{2+}$ levels which elicit the exocytosis of cytoplasmic membrane vesicles containing Trk receptors (Du et al., 2000; Meyer-Franke et al., 1998).

The key event for RTK-mediated signalling is the recruitment of proteins through the SH2 (Src homology 2) and PTB (phosphotyrosine binding) domains (Segal and Greenberg, 1996). Thus, phosphorylated tyrosine 490 (pY490) on TrkA (or pY484 on TrkB) serves as a docking site for the SH2 and PTB-containing adapter proteins Shc (Src/α-collagen) and Grb2 (growth factor receptor-bound protein 2) for subsequent activation of the Ras-mediated ERK 1&2 pathway (Huang and Reichardt, 2003). Phosphorylation of tyrosine 490 and 484 (on TrkA and TrkB respectively) has also been shown to activate the phosphoinositol-3-kinase (PI3K) signalling pathway where PI3K can be activated by the small GTP-binding protein Ras (Patapoutian and Reichardt, 2001; Gunn-Moore and Tavare, 1998). In addition, phosphorylated Grb2 recruits adapter proteins Gab which results in the recruitment and activation of PI3K

(Holgado-Madruga et al., 1997; Kaplan and Miller, 2000). Furthermore, PI3K can be activated through Ras-independent pathway following the phosphorylation of tyrosine 751 (pY751) on TrkA and this subsequently results in the activation of downstream serine/threonine kinase Akt or protein kinase B (PKB) (Huang and Reichardt, 2003; Gunn-Moore and Tavare, 1998). Phosphorylation of tyrosine 785 (pY785) of TrkB has also been shown to be essential for the activation of phospholipase C γ (PLCγ) where it forms the binding site for SH2 domain of PLCγ to cause activation of the phospholipase (Gunn-Moore and Tavare, 1998).

Neurotrophin-mediated signalling is implicated in a wide range of neuronal functions, including cell survival, proliferation, axon and dendrite growth and differentiation (Huang and Reichardt, 2003). The BDNF-mediated signalling is of pertinence to this thesis since BDNF has been shown to induce ERK 1&2-dependent phosphorylation of synapsin, thereby resulting in an increase in glutamate release from cerebrocortical synaptosomes (Jovanovic et al., 2000). However, whether BDNF effects the activation of ERK 1&2 underlying the control of presynaptic function through the activation of Ras is unclear. Previously, studies have shown several possibilities where neurotrophin may activate excitability of the cell by activating $Na^+$ channels (Urbano and Buno, 2000; Kafitz et al., 1999) and/or by enhancing intracellular $Ca^{2+}$ levels (Baldelli et al., 2000; Li et al., 1998; Stoop and Poo, 1996). Thus, one of the objectives of this thesis is to identify the signalling cascade and mediators in the BDNF-mediated ERK 1&2 activation in nerve terminals.

### 1.3.4. Non-receptor tyrosine kinases (nRTKs)

The Src family protein kinases (SFKs) is a family of non-receptor tyrosine kinases (nRTKs) that play key roles in regulating signal transduction by a diverse set of cell surface receptors (Parsons and Parsons, 2004; Kalia et al., 2004; Salter and Kalia, 2004; Crossthwaite et al., 2004; Luttrell et al., 1996). SFKs were originally defined following the discovery of v-Src, a viral protein encoded by avian tumour-causing

oncogene from Rous sarcoma virus (Martin, 2001). Studies on Rous sarcoma virus later yielded the human cellular homologue encoded by the cellular proto-oncogene, c-Src. Altogether, there are 11 members of the SFKs in humans: Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm and Yes. Members of SFKs all contain a membrane-targeting N-terminal segment SH2 and SH3 domains (Roskoski, Jr., 2005; Roskoski, Jr., 2004; Parsons and Parsons, 2004). Both the SH2 and SH3 domains are important sites that mediate interactions with target substrates containing phosphotyrosine and proline-rich motifs. Crystallographic analysis revealed an autoinhibitory domain in SFKs which locks the kinase in an inactive conformation (Roskoski, Jr., 2005; Parsons and Parsons, 2004). Activation of the Src kinase can be achieved by protein tyrosine phosphatases, such as PTPα, which mediate dephosphorylation on pTyr527. In addition, full activation also requires autophosphorylation of the Src kinase at Tyr 416, which is present in activation loop to promote kinase activity (Salter and Kalia, 2004; Xu et al., 1999). As SFKs act to regulate signals from cell surface receptors, their activities are controlled by RTKs, integrin receptors, G-protein coupled receptors (GPCRs), antigen, cytokine receptors and steroid receptors and crosstalking between these pathways can be readily seen (Thomas and Brugge, 1997). In several systems, studies have also demonstrated the activation of the SFKs through a $Ca^{2+}$-dependent mechanism (Ginnan and Singer, 2002; Rocic et al., 2001; Perkinton et al., 1999; Siciliano et al., 1996; Lev et al., 1995; Rusanescu et al., 1995).

SFKs are found widely expressed in neurons (Boxall and Lancaster, 1998) where they have been implicated in cell growth, differentiation, cell morphology, migration and survival, as well as controlling the turnover of cell surface receptors and modulating actin cytoskeleton arrangements (Hoffman-Kim et al., 2002; Kuo et al., 1997; Wang and Salter, 1994). In the postsynaptic terminal, SFKs have been primarily found to be involved in up-regulating the activities of NMDA receptors and other ion channels that mediate fast excitatory transmission (Kalia et al., 2004; Salter and Kalia, 2004; Fadool et al., 1997; Cataldi et al., 1996). Recent findings also indicated an

involvement of SFKs in mediating phosphorylation of neurotransmitter receptors such as nACh-R (Sadasivam et al., 2005) and AMPA receptors (Hayashi and Huganir, 2004). Presynaptically, SFKs are associated with the SVs and account for the majority of synaptic vesicle tyrosine kinase activity (Linstedt et al., 1992). On the synaptic vesicle, the SFK Src has been demonstrated to associate with synapsin through its SH3 domain, suggesting a role of SFKs in regulating neurotransmitter release (Foster-Barber and Bishop, 1998; Onofri et al., 1997). Indeed, studies have shown that Src kinase is triggered by $Ca^{2+}$ influx to induce tyrosine phosphorylation of several proteins, including synaptophysin (Barnekow et al., 1990). A facilitatory role of Src kinase in regulation of glutamate release from cerebrocortical synaptosomes has also been shown previously (Wang, 2003), although studies in PC12 cells and primary cultured neurons have yielded an opposing result where Src kinases appear to play an inhibitory role in neurotransmitter release (Ohnishi et al., 2001). Thus, this lack of agreement of the function of SFKs in the control of presynaptic function warrants further investigation. One aspect of this thesis is, therefore, concerned with studying the role of SFKs in the modulation of presynaptic ERK 1&2 signalling following neuronal stimulation.

### 1.4. Modulation of synaptic function by G-protein coupled receptors

From the discussion so far, the importance of $Ca^{2+}$ in the regulation of presynaptic functions is apparent given that $Ca^{2+}$ can initiate activation of various protein kinases or phosphatases as well as signalling mediators to bring about specific neuronal responses. Presynaptically, a large amount of evidence suggests that presynaptic function can also be modulated by heterotrimeric G-protein coupled receptors (GPCRs) activation which results in altering the amount of $Ca^{2+}$ levels in the cell to mediate facilitatory or inhibitory effects. Facilitatory GPCRs, like the group I metabotropic glutamate receptors (mGluR1 and mGluR5), have been shown to increase $Ca^{2+}$ levels by modulating $Ca^{2+}$ release from intracellular stores through a phospholipase C (PLC)-dependent mechanism following $G\alpha_q$ activation (Pin and

Duvoisin, 1995), and have been implicated in the increase of glutamate release from nerve terminals (Herrero et al., 1998). On the other hand, inhibitory GPCRs modulate the activities of VGCCs to control the amount of extracellular $Ca^{2+}$ influx through the membrane-delimited $G_{\alpha i/o}$ protein (Gutkind, 2000). These include the adenosine ($A_1$) receptors and γ-aminobutyric acid ($GABA_B$) receptors. Studies have shown that activation of $G_{\alpha i}$-coupled $A_1$ receptors results in the inhibition of presynaptic function by exerting regulation on VGCC activities (Ribeiro et al., 2002). Given that this thesis addresses presynaptic modulation of ERK 1&2 signalling by the $GABA_B$ receptor activation, $G_{i/o}$ coupled-$GABA_B$ receptor is reviewed.

$GABA_B$ receptors are metabotropic GPCRs that mediate slow inhibitory transmission via heterotrimeric $G\alpha_{i/o}$ proteins (Bowery, 1989). $GABA_B$ receptors belong to the so-called 'family 3' of GPCRs, which has close homology to the metabotropic glutamate receptor (mGluR), the calcium-sensing receptor (Couve et al., 2000; Wise et al., 1999), and a group of putative taste receptors (Hoon et al., 1999). Functional $GABA_B$ receptors are heterodimers composed of $GABA_BR1$ and $GABA_BR2$ subunits, where $GABA_BR1$ subunit contains the ligand binding site (Galvez et al., 1999), while the $GABA_BR2$ subunit links to the pertussis toxin-sensitive $G_i/G_o$ protein (Margeta-Mitrovic et al., 2001) and is required for cell surface trafficking (Balasubramanian et al., 2004).

In the rat brain, $GABA_B$ receptors trigger inhibition of synaptic transmission at both post- and pre-synaptic locations. Postsynaptically, $GABA_B$ receptor activation has been shown to activate multiple $K^+$ channel subtypes, leading to slow inhibitory postsynaptic potentials (IPSPs) (Nicoll, 2004; Luscher et al., 1997). In conjunction with this postsynaptic inhibitory role, activating presynaptic $GABA_B$ receptors also showed suppression of neurotransmitter release, including glutamate and GABA (Harvey and Stephens, 2004; Perkinton and Sihra, 1998; Pende et al., 1993). Studies

demonstrated that activation of presynaptic $GABA_B$ receptors by baclofen, a $GABA_B$ receptor agonist, leads to a reduction in electrically evoked neurotransmitter release in guinea-pig cerebrocortical slices (Potashner, 1979) and rat cerebrocortical synaptosomes (Perkinton and Sihra, 1998; Pende et al., 1993; Bonanno and Raiteri, 1992). More recent studies have reported on the mechanisms involved in these $GABA_B$-mediated effects where inhibition of glutamate release by baclofen was found to be dependent on decreased conductance of the VGCC coupled to exocytosis (Nicoll, 2004; Perkinton and Sihra, 1998; Dittman and Regehr, 1996). In addition, presynaptic $GABA_B$ receptors may act to hyperpolarise neurons through activation of $G_{i/o}$ coupled to inwardly rectifying $K^+$ channels (Kubota et al., 2003). There is also evidence that $GABA_B$ receptors are negatively coupled to AC through $G_{i/o}$ to inhibit cAMP production as well as modulating the activity of PKC (Kubota et al., 2003; Bowery et al., 2002; Sakaba and Neher, 2003; Mott and Lewis, 1994). A feedback regulatory system is also believed to operate where $GABA_B$ receptors may be regulated by the activities of PKC and PKA (Gomez-Villafuertes et al., 2004; Perkinton and Sihra, 1998).

### 1.5. Regulators of ERK 1&2 signalling

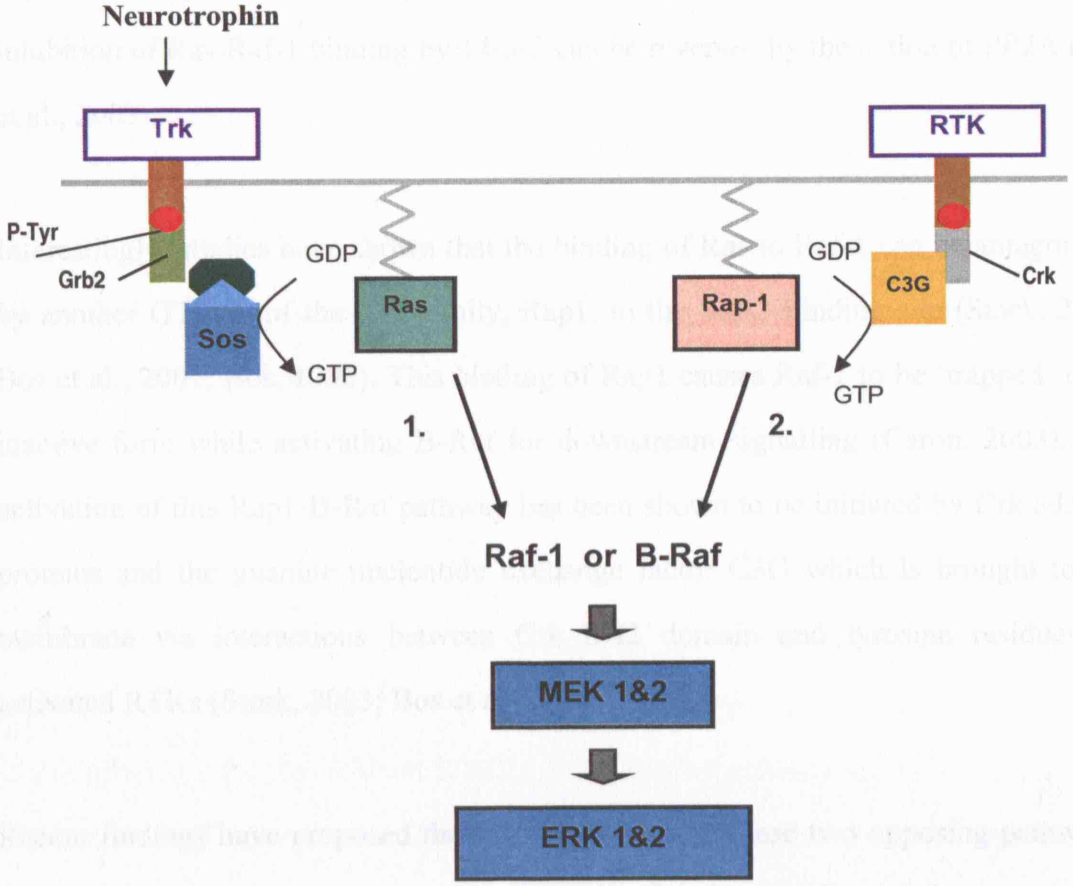
As discussed above, the activation of ERK 1&2 is important in the regulation of both short-term and long-term synaptic plasticity. Clearly, the ERK signalling pathway must be tightly controlled in its mechanism of activation/inactivation, its duration of activation as well as subcellular localisation to ensure the proper outcome of responses. Thus, this thesis is concerned with the elucidation of the upstream regulation and crosstalk of ERK 1&2 signalling pathways underlying presynaptic modulation. The introduction will further focus on different aspects of upstream regulation of ERK 1&2 activation, including regulation by kinases and phosphatases, activation during $Ca^{2+}$ and neurotrophic stimulation, factors that determine the fidelity and spatio-temporal activities of ERK 1&2, as well as crosstalk of signalling pathways leading to ERK 1&2 activation.

### 1.5.1. Upstream activation of ERK 1&2

Like other MAPKs, the activation of ERK 1&2 involves a cascade consisting of three sequential kinase reactions, which result in the activation of proteins by promoting phosphorylation specifically, thus enabling the initial signal to be amplified and fine-tuned. ERK 1&2 is activated by the dual phosphorylation of a threonine and tyrosine residue in its activation loop (T202/Y204) by the MAPK kinases (or MKKs) known as MEK 1&2. These kinases are, in turn, activated by phosphorylation brought about by upstream serine/threonine-specific MAPK kinase kinase (MKKK) also known as Raf (Wellbrock et al., 2004; Chang and Karin, 2001).

In mammals, there are three Raf proteins, namely Raf-1 (or c-Raf), A-Raf and B-Raf. These Raf proteins differ in their pattern of tissue expression and their mechanisms of activation. Raf-1 and B-Raf mRNA are found expressed in the brain whereas detection of A-Raf is restricted to urogenital tissue (Wellbrock et al., 2004; Hagemann and Rapp, 1999). Subcellular localisation studies further revealed that whilst both Raf-1 and B-Raf are found in the cytoplasm, Raf-1 is localised mainly in the cytosolic fraction around the nucleus and B-Raf is widely distributed in neuritic extensions (Morice et al., 1999).

Apart from the differential localisation, the Raf proteins are differentially regulated by upstream kinases which can then lead to the differential modulation of the ERK 1&2 signalling cascade **(Schematic 1.5.1)**. The activation of Raf-1 by Ras in the ERK 1&2 signalling pathway has been well documented over the years and has been implicated in neuronal functions such as cell survival and differentiation (Wellbrock et al., 2004; Bos, 1998). Ras is a small GTP-binding protein that binds to the N-terminal of Raf-1 and, in so doing, recruits Raf-1 to the plasma membrane to allow activation in a phosphorylation-dependent manner (Wellbrock et al., 2004). The ability of Raf-1 to




**Schematic 1.5.1. The Raf proteins involved in mediating ERK 1&2 activation.** 1.) Neurotrophin binding to Trk receptors leads to autophosphorylation of tyrosine residues on the RTK. This results in the recruitment of adaptor Grb2 and nucleotide exchange factor Sos to cause the activation of Ras. Ras then activates downstream Raf-1 or B-Raf to cause subsequent MEK 1&2 and ERK 1&2 activation; 2.) Activation of RTK recruits the adaptor Crk and nucleotide exchange factor C3G to cause the activation of Rap1. Rap1 then activates downstream B-Raf to subsequently cause activation of MEK 1&2 and ERK 1&2. Brown = RTK; red = phosphotyrosine residues on RTK.

bind to Ras has been shown to depend upon the regulation by the 14-3-3 adaptor/scaffold proteins which bind Raf-1 through specialised phosphorylated peptide motifs, thus, interfering with Raf-1 binding to Ras (Light et al., 2002). This inhibition of Ras-Raf-1 binding by 14-3-3 can be reversed by the action of PP2A (Ory et al., 2003).

Interestingly, studies have shown that the binding of Ras to Raf-1 can be antagonised by another GTPase of the Ras family, Rap1, to the Raf-1 binding site (Stork, 2003; Bos et al., 2001; Bos, 1998). This binding of Rap1 causes Raf-1 to be 'trapped' in its inactive form while activating B-Raf for downstream signalling (Caron, 2003). The activation of this Rap1-B-Raf pathway has been shown to be initiated by Crk adaptor proteins and the guanine nucleotide exchange factor C3G which is brought to the membrane via interactions between Crk SH2 domain and tyrosine residues on activated RTKs (Stork, 2003; Bos et al., 2003).

Recent findings have proposed the functional roles of these two opposing pathways, where Ras and Rap-1 act largely independently of each other. Reports have suggested that while transient activation of ERK 1&2 signalling requires mediation through the Ras-Raf1-mediated pathway, Rap1-dependent B-Raf pathway is essential for sustained and maximal phosphorylation of ERK 1&2 (York et al., 1998). The activation of these two pathways has been shown to be regulated differentially by upstream kinases such as PKA (Wellbrock et al., 2004). Reports have indicated that PKA inhibits Raf-1 activation by phosphorylating Raf-1 to disrupt the Ras-Raf-1 interaction, leading to an inhibition of ERK 1&2 activation. On the other hand, an enhancement in B-Raf activation has been observed with cAMP or PKA whereby ERK 1&2 activation is increased (Vossler et al., 1997). Moreover, there is evidence to suggest that regulation by activated Src also occurs differently with the two pathways. Whereas Ras-Raf-1 signalling cascade can be stimulated by a mechanism dependent on the activation of Src, the B-Raf pathway is not responsive to activated Src, but has

been found constitutively active (Wellbrock et al., 2004).

Whilst the above data suggest a model for Rap1-B-Raf functioning as a repressor of Ras-Raf-1 signalling and being the major player in sustained ERK 1&2 activation, the exact roles of these two pathways are still not fully appreciated with studies showing both potentiating and antagonising effects of Ras-Raf-1 and Rap1-B-Raf in the regulation of ERK 1&2 (Stork, 2003; Bos et al., 2003; Bos et al., 2001; Zwartkruis and Bos, 1999). The ability of Rap1 and Ras to regulate ERK 1&2 may, therefore, depend upon the cell type and the availability of Ras, Rap1, Raf-1 and B-Raf, as well as their regulators (Stork, 2003).

Unlike Raf kinases, the Ras family members are highly conserved with numerous effectors and upstream regulators such as guanine nucleotide exchange factors (GEFs) and GTP-activating protein (GAP) controlling their functions. Ras is activated by Sos which promotes the dissociation of GDP from Ras in exchange for GTP. In addition, GEFs such as Ras GRF have also been found to activate Ras, whereby activities of these GEFs require the binding of $Ca^{2+}$/CaM and $Ca^{2+}$/diacylglycerol (DAG) respectively ((Zwartkruis and Bos, 1999), see the next section 1.5.2). Conversely, the activity of Ras is negatively controlled by p120-Ras-GAP, a GTPase-activating protein, which acts to hydrolyse Ras-GTP to Ras-GDP. As well as targeting Raf for downstream ERK 1&2 signalling, Ras proteins also can bind to and activate other effectors, such as PI3Ks to elevate levels of PtdIns(3,4,5)$P_3$ ($IP_3$) and Akt/PKB activities (Marshall, 1996).

### 1.5.2. Factors affecting the signalling specificity of ERK 1&2

Given the diversity of cellular effects mediated by ERK 1&2 signalling, it is important that mechanisms exist to regulate the specificities of the cascade. Indeed, neurons have a number of ways to control the fidelity and spatio-temporal activation of ERK 1&2 by: 1) the presence of scaffold proteins or docking proteins. 2)

subcellular compartmentalisation. 3) MAPK phosphatases (MKPs). 4) Upstream Ras and Rap1 modulators such as $Ca^{2+}$ and CaM.

### 1.5.2.1. Scaffold and docking proteins

The fidelity of ERK 1&2 signalling is ensured by a number of scaffold proteins which act to generate multienzyme complexes to bring together components of a single kinase cascade (Torii et al., 2004; Pouyssegur and Lenormand, 2003). In mammalian cells, the best example is the MEK partner 1 (MP1) and p14 which complex together to scaffold MEK1 and ERK1 to facilitate their activation (Wunderlich et al., 2001; Schaeffer et al., 1998). Another scaffold protein called kinases suppressor of Ras (KSR) was also identified to directly interact with MEK and ERK via distinct domains, causing translocation of the Ras-mediated signalling module to the cell membrane in response to growth factor treatment (Morrison, 2001). Recently, the dephosphorylation of KSR1 and Raf-1 has been shown to be mediated by PP2A resulting in increased Ras-ERK activation (Ory et al., 2003). MAPKs also contain docking sites which enable localisation of kinases to favour activation of proteins at specific sequences (Pouyssegur and Lenormand, 2003). For example, an acidic cluster known as CD (common docking site) is found on ERK 1&2 where this docking site is localised opposite to the kinase catalytic cleft to which substrates or ERK-interacting proteins bind for subsequent activation by the kinase. This docking groove may contain several interacting motifs that cooperate with each other to increase the affinity of binding for the ERK-interacting proteins. Thus, depending on the number, type, position and arrangement of these docking sites, the specificity of an individual interaction in the ERK pathway can be ascertained.

### 1.5.2.2. Spatial localisation of proteins

The duration and intensity of the ERK 1&2 response are, in part, controlled by spatial localisation (Torii et al., 2004; Pouyssegur and Lenormand, 2003). Data suggest that Raf-1, in its inactive state, is normally located in the cytosol and that during

stimulation, the translocation of Raf-1 to the plasma membrane is essential for the activation of Raf-1 by Ras (Wellbrock et al., 2004; Marais et al., 1997). This is supported by the demonstration that the Ras interaction with Raf-1 is not sufficient to fully activate the Raf protein unless Ras-GTP is membrane bound (Marais et al., 1997). In addition, Raf may be localised to different cellular components to activate different signal responses. For instance, localisation of Raf (MEK kinase) to cytoskeletal elements has been shown to play a role in cell motility modulation (Yujiri et al., 1998). Furthermore, the spatial localisation of various MAPK kinases and phosphatases (such as PP2A and MKPs) also plays an important part in contributing to the specificity of ERK response.

### 1.5.2.3. MAPK phosphatases (MKPs)

The temporal regulation of ERK signalling can be finely controlled by the presence of MAPK kinases as well as MAPK phosphatases (MKPs). Whereas MAPK-specific kinases positively modulate the activity of MAPK signals, MAPKs are negatively regulated by MKPs. MKPs are dual specific phosphatases that cleave phosphodiester bonds in substrates containing phosphotyrosine as well as phosphoserine and phosphothreonine residues. There are at least eight known members of the MKP family currently identified (Camps et al., 2000), three of which, MKP 1, 2 and 3 are phosphatases that are capable of dephosphorylating ERK 1&2. Previous studies showed that MKPs are not expressed in quiescent cells and may be induced upon stimulation by agonists such as stress, mitogens and UV irradiation (Camps et al., 2000). Examinations of phosphatase activities *in vitro* showed that MKP 1 and 2 expression can be stimulated by expression of Ras (or MEK) alone, thus, indicating that activation of ERK 1&2 cascade is sufficient to promote expression of MKP 1 and 2 for an inhibitory feedback loop (Brondello et al., 1997). Indeed, Ser359 and Ser364 on MKP1 have been shown to be phosphorylated by ERK. This does not modify the phosphatase activity, but increases the half-life of MKP1, to thereby reinforcing the negative feedback control (Pouyssegur and Lenormand, 2003; Brondello et al., 1999).

There is evidence to suggest that MKPs are differentially localised to different subcellular compartments in the cell (Pouyssegur and Lenormand, 2003; Camps et al., 2000). Whereas MKP 1 and 2 are primarily expressed in the nucleus, MKP 3 has been shown to be restricted to the cytoplasm. However, the precise role of these MKPs is still unclear *in vivo* and requires further elucidation.

### 1.5.2.4. Regulation of Ras-GEFs and GAPs by second messengers

As well as MAPK kinases and phosphatases, the spatio-temporal regulation of guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) also coordinates the Ras/Rap1 signalling events. Studies have shown that these GEFs and GAPs are ultimately activated by second messengers such as $Ca^{2+}$ and diacylglycerol (DAG), levels and spatio-patterns of which alter in response to particular stimuli. There is evidence that PKC, which is activated by increased cytosolic $Ca^{2+}$ levels and DAG, can phosphorylate Raf-1 leading to increased ERK activation (Aspenstrom, 2004; Walker et al., 2003; Zwartkruis and Bos, 1999). $Ca^{2+}$ can also affect Ras activities through direct regulation on EF-hand domains-containing GEF (Walker et al., 2003; Ebinu et al., 1998; Farnsworth et al., 1995). In addition, GAPs contain C2 domains which are $Ca^{2+}$ and phospholipid-binding, and thereby, cause Ras/Rap1 deactivation upon $Ca^{2+}$-stimulated recruitment to the membrane (Aspenstrom, 2004; Walker et al., 2003). This suggests a direct and dynamic $Ca^{2+}$-dependent regulation of Ras/Rap1 cycling in ERK 1&2 signalling. Furthermore, $Ca^{2+}$/CaM has been demonstrated to directly regulate the K-isoform of Ras, although this has been shown to result in reduced ERK 1&2 signalling (Villalonga et al., 2001). The suggestion of $Ca^{2+}$-dependent activation of ERK 1&2 has also come from studies showing that ERK 1&2 activation can be facilitated with elevated levels of intracellular $Ca^{2+}$ levels as a result of either depolarisation-induced $Ca^{2+}$ influx or ionotropic receptor activation (Baldassa et al., 2003; Agell et al., 2002; Finkbeiner and Greenberg, 1996; Rosen et al., 1994), as well as direct entry of $Ca^{2+}$ effected by the use of $Ca^{2+}$ ionophores in smooth vesicular muscle (Ginnan and

Singer, 2002).

### 1.5.3. Cross-talk of signalling pathways leading to ERK 1&2 activation

While growth factor-mediated stimulation of Trk-Ras pathway represents the major route for stimulation of ERK 1&2 (see section 1.3.3), increasing amounts of evidence indicate that activation of ERK 1&2 can occur as a result of cross-talk between signalling pathways. Several groups have shown that the GPCR-mediated pathways can cross-talk with the ERK 1&2 pathway to generate a cellular response (Vanhoose et al., 2002; Gutkind, 2000; Lopez-Ilasaca, 1998).

The transactivation of RTK following $G_{i/o}$ activation is thought to be mediated by Gβγ subunits from studies showing that overexpression of Gβγ can stimulate Ras-dependent ERK activation in Cos-7 cells (Crespo et al., 1994). Thus, free Gβγ may affect the function of Ras by binding the PH-domain (plecktrin homology domain which also bind to phosphoinositides) of one or more Ras-regulatory proteins, to recruit Ras to the membrane where it can then be activated (Lemmon et al., 1996). Although the mediators of this RTK transactivation is still unclear, some evidence poses a number of kinases, including Src family protein kinases, PI3K and PKC as intermediates in the pathway (Gutkind, 2000; Lopez-Ilasaca, 1998).

The role of Src family non-receptor tyrosine kinases has been directly evaluated recently. Studies have shown that activation of c-Src is involved in Gβγ-mediated stimulation of ERK 1&2 pathway where Shc, a protein interacting with the Sos-Grb2 complex downstream of Trk receptor, became phosphorylated by the SFK (Luttrell et al., 1996). How Src kinases are activated in response to GPCR agonists is not fully understood, but studies on the mechanism by which β-adrenergic receptors stimulate ERK cascade provide some insights (Tohgo et al., 2003; Gutkind, 2000; Luttrell et al., 1999). In this case, binding of agonist to the β-adrenergic receptor causes

phosphorylation of the receptor by GPCR kinase (GRK) and the subsequent recruitment of β-arrestin, which functions as an adaptor to recruit Src kinases to the membrane for their activation. This activation and the subsequent internalisation of the β-arrestin and Src kinase complex has been shown to play an essential part in the activation of ERK 1&2 (Tohgo et al., 2003; Gutkind, 2000; Luttrell et al., 1999). In some studies, SFKs have been shown to directly associate with RTK, where Src can be activated by RTK (Iwasaki et al., 1998; Bromann et al., 2004). Other reports also suggest PI3K to be a linker between GPCR and ERK 1&2 signalling with experiments showing the action of PI3K downstream of Gβγ and upstream of the Src family kinases, although the mechanism of this interaction is still unclear (Pleiman et al., 1993).

Apart from Src kinases, ERK 1&2 activation by $G\alpha_i$ and/or $G\alpha_q$ proteins has also been shown to be mediated by another member of the nRTK family, Pyk2, which is a $Ca^{2+}$-dependent kinase. In this pathway, integrin binding initiates GPCR signalling where Pyk2 is recruited and activated by the increase in $Ca^{2+}$ levels as a consequence of PLC activation by $G\alpha_q$ (Della Rocca et al., 1997; Dikic et al., 1996). This subsequently causes the recruitment and phosphorylation of intracellular complex including focal adhesion kinase (FAK). Activated FAK, then, provide docking sites for other proteins such as Src, Grb2and Sos to mediate GPCR signalling to Ras-MEK-ERK cascade (Giancotti and Ruoslahti, 1999).

Stimulation of ERK 1&2 signalling by $G\alpha_q$-coupled receptors involves the activation of phospholipase C (PLC) and produces the downstream second messengers DAG (diacylglycerol) and $IP_3$ (inositol-1,4,5-triphosphate) (Gutkind, 2000). Thus, $IP_3$ mobilises intracellular $Ca^{2+}$ whilst DAG, together with $Ca^{2+}$, activates a family of $Ca^{2+}$/phospholipid-dependent protein kinase known as protein kinase C (PKC). PKC can then directly phosphorylate Raf-1 to activate downstream MEK 1&2 (Kolch et al.,

1993). This positive modulatory role of ERK 1&2 signalling by PKC has been shown in a number of systems, including cerebrocortical synaptosomes (Brodie et al., 1999; Roberson et al., 1999; Davies, 2004). However, recent studies have found that phosphorylation of Raf-1 by PKC is not sufficient to increase Raf-1 phosphorylation of MEK (Macdonald et al., 1993). Thus, rather than offering a Ras-independent pathway to ERK 1&2 signalling, PKC-mediated phosphorylation of Raf-1 may act to facilitate full activation of Raf-1 once it is bound to Ras (Burgering and Coffer, 1995). Apart from mediating activation of ERK through a PKC-dependent pathway, $G_q$-coupled receptors can also activate ERK 1&2 in a PKC-independent manner. This can be achieved through the activation of PLC, which stimulates the production of second messengers $IP_3$ to release $Ca^{2+}$ to directly bind to and stimulate the upstream regulators of Ras-signalling, such as Ras-GRF (English et al., 1999).

ERK 1&2 activation can also be mediated via $G\alpha_s$ where stimulation of GPCRs is coupled to increased adenylyl cyclase (AC) activity. The resulting increase in cAMP and subsequent activation of PKA enables modulation of ERK 1&2 activation at the level of Raf-1, because PKA is able to phosphorylate Raf-1 to cause inhibition of the ERK 1&2 pathway ((Vossler et al., 1997; Wu et al., 1993), see section 1.5.1). On the contrary, facilitation of ERK 1&2 can also occur when PKA phosphorylates Rap-1. Indeed, studies in rat cerebral cortex have revealed that stimulation of PKA by cAMP following β-adrenoreceptor activation or direct stimulation of AC by forskolin can both result in a substantial increase in the activation of ERK 1&2 (Davies, 2004). These data suggest a mechanism by which PKA can crosstalk and activate or inhibit ERK 1&2 signalling via Gα activation. In addition to the PKA-dependent pathway, recent reports have also suggested signalling through Gαs can be mediated through the activation of Raf-1 by Src, although the mechanism is yet to be determined (English et al., 1999).

Apart from the positive regulation of ERK 1&2 activation by GPCR, studies indicate a negative feedback mechanism also exists where ERK has been shown to be negatively regulated by the activity of GPCR kinase 2 (GRK2) which controls the activation of GPCRs (Pitcher et al., 1999). There is also evidence that the $G\alpha_{i/o}$-coupled $GABA_B$ receptor activation can attenuate ERK 1&2 activation (Ren and Mody, 2003). However, in some cases, this $GABA_B$-mediated negative modulation of ERK 1&2 signalling has not been observed. In these studies, increased ERK 1&2 phosphorylation was demonstrated following the addition of $GABA_B$ receptor agonist baclofen (Vanhoose et al., 2002). Thus, the contradictory effects of $GABA_B$ receptor activation in the regulation of ERK 1&2 signalling require further elucidation.

From the above discussion, it was interesting to note that the activation of ERK 1&2 by neurotrophins (such as BDNF-mediated pathway, see section 1.3.3) and many of the GPCRs (such as PKA/PKC-dependent, PI3K/PLC-dependent, $GABA_B$ receptor activation-dependent and Src kinase-dependent pathways) involve the activation of protein kinases or mediators that respond to changes in intracellular levels of $Ca^{2+}$. Having previously discussed that ERK 1&2 activation is an activity-dependent process that plays an important role in the control of presynaptic plasticity, the regulation of ERK 1&2 activation by $Ca^{2+}$, both directly and indirectly through other modulators, is the issue in the current work.

### 1.6. Models for examination of presynaptic function

The aim of this thesis is to examine the upstream regulation of ERK 1&2 activation underlying neurotransmitter release. Over the last decade, a number of preparations have been available to study the regulation of neurotransmitter release. Brain slices were considered good preparations to study regulation of release as they maintain intact neuronal circuitry that enabled release from neurons to be electrically stimulated under physiological conditions (Collingridge, 1995). However, as brain slices contain complete neuronal and glial cells, the study of regulation of release by

presynaptic receptors cannot be examined in isolation in these preparations. Like the brain slices, the fact that neuronal cell cultures are still attached to the cell bodies and are impinged upon by various post-synaptic effect, also makes evaluation of presynaptic effects complicated.

### 1.6.1. Isolated nerve terminals (synaptosomes)

Studies with guinea-pig and rat brain have shown that synaptosomes are good models for investigating the characteristics of neurotransmitter release in the mammalian nervous system (Rodriguez-Moreno and Sihra, 2004; Jovanovic et al., 2000; Nicholls and Coffey, 1994; Sanchez-Prieto et al., 1987; Nicholls and Sihra, 1986). Synaptosomes are isolated nerve terminals that are of 0.5-1μm in diameter that maintain features of intact nerve terminal, both bioenergetically and functionally (Maycox et al., 1990; Whittaker and Dowe, 1964; Gray and Whittaker, 1962). For example, studies showed that compartments such as small synaptic vesicles containing neurotransmitters and, in some cases, mitochondria can be observed in isolated brain synaptosomal homogenates. There is also evidence that synaptosomes are capable of respiring and producing ATP, as well as maintaining a plasma membrane potential in media containing a low concentration of external $K^+$ (Nicholls, 1989). In addition, studies have shown that synaptosomes can maintain physiological levels of cytosolic $Ca^{2+}$, and depolarisation-dependent increases can be achieved by the use of biochemical reagents such as 4-aminopyridines (4AP) and external $K^+$ (KCl) (Nicholls and Coffey, 1994; Sihra et al., 1992; McMahon and Nicholls, 1991b). As an alternative means of stimulating neurotransmitter release, direct entry of $Ca^{2+}$ can be mediated with the use of $Ca^{2+}$ ionophores ionomycin to bypass modulation of membrane excitability and $Ca^{2+}$ entry via VGCCs (Sihra et al., 1992; McMahon and Nicholls, 1991b). Since synaptosomes are devoid of components such as the cell body, the axon and any post-synaptic functions, they represent a useful and well established model for studying presynaptic regulatory pathways for neurotransmitter release and their crosstalk, without the complications of interpretation of postsynaptic

events. Although the synaptosomal preparation is heterogeneous by its nature, the majority of the nerve terminals are glutamatergic and GABAergic. Thus, the signalling mechanisms observed in this preparation mainly reflect events occurring in these predominating nerve terminals.

### 1.7. PhD Aims

- To understand the molecular pathways upstream of ERK 1&2 underlying the regulation of release in cerebrocortical nerve terminals.

- To evaluate the involvement of kinases and phosphatases in the regulation of ERK 1&2 activation.

- To identify and characterise key mediators and regulators of presynaptic ERK 1&2 signalling.

- To investigate the cross-talk between distinct signalling pathways in the regulation of ERK 1&2 activation.

# Chapter 2

# MATERIALS AND METHODS

# 2. MATERIALS AND METHODS

## 2.1. Introduction to synaptosomal preparation for rat brain

The protocol for the preparation of synaptosomes from rat brain has been described extensively (Sihra, 1997), involving steps of homogenisation, differential centrifugation and purification by density gradients. Whilst the centrifugation steps were primarily used to remove heavy non-neuronal elements, the density gradients were used to remove free mitochondria, myelin and light membranes from the synaptosomal suspension. The density gradient was first developed using sucrose gradients (Whittaker, 1968). However, as sucrose gradients expose synaptosomes to very hypertonic conditions, the technique was not suitable for obtaining synaptosomes with good metabolic properties. The Ficoll gradients, based on a polysaccharide polymer, were subsequently established (Verity, 1972). Although Ficoll-gradient-purified synaptosomes appear to be metabolically more competent, these synaptosomes are more heterogenous than those purified by the sucrose gradients. Most recently, Percoll gradient-based purification has been developed and has several advantages over conventional density gradients. Percoll gradients are based on non-toxic polymer beads and are iso-osmotic. These properties effect the best synaptosomal purification yielding preparations with high metabolic competency without the effects of dehydration (Dunkley et al., 1986; Nagy and Delgado-Escueta, 1984). Furthermore, Percoll beads can also be easily washed away from membranes by simple centrifugation after the purification step. In the next section, we describe in details the protocols for the synaptosomal preparation which utilise this Percoll density gradient for the purification of the nerve terminals.

### 2.1.1. Protocols for synaptosomal preparation

Synaptosomes were prepared from cortices of 2-months old (150-200g) male Sprague-Dawley rats as described previously (Sihra, 1997). Rats were killed by stunning and decapitation according to procedures covered under the Home Office

Animals (Scientific Procedures) Act of 1986. The brain was rapidly removed and cerebral cortices carefully dissected and homogenised in 320mM sucrose (sucrose medium) with a Potter-Elvejhem tissue grinder consisting of a smooth glass mortar and Teflon pestle (0.1-0.15mm clearance) rotated at 900rpm using 8-10 up/down strokes. The homogenate was centrifuged at relative centrifugal force (RCF) of 3,020xg for 2 min at 4°C. The $P_1$ pellet, containing connective tissue, blood vessels, cell body, nuclei and myelin, was discarded and the supernatant ($S_1$) was transferred to fresh tubes and re-centrifuged at RCF 14,600xg for 11 min at 4°C. The supernatant ($S_2$) was then discarded and the pellet ($P_2$) (representing the crude synaptosomal suspension) was kept. As the $P_2$ pellet was contaminated with free mitochrondria, myelin and other light membranes, the suspension was then purified using Percoll gradients. To do this, the $P_2$ pellet was resuspended in 2ml sucrose medium with a hand-operated Dounce (glass pestle and tube) homogeniser. The total volume was made up to 8ml with the sucrose medium and the suspension was mixed by gentle inversion of the tube. Percoll gradients were prepared by layering 2.5ml of 23%, 10% and 3% Percoll into 4 x polycarbonate tubes using a peristaltic pump (see Table 2.1.1 for preparation). Two millilitres of the resuspension was layered on top of each Percoll gradient and tubes were centrifuged at RCF 35,100xg for 6 min at 4°C.

| %(v/v) Percoll | ml of filtered Percoll | ml of 5x Sucrose | µl of 0.5M EDTA | µl of 0.1M DTT | ml of water |
|---|---|---|---|---|---|
| 23% | 23 | 20 | 200 | 250 | 57 |
| 10% | 10 | 20 | 200 | 250 | 65 |
| 3% | 3 | 20 | 200 | 250 | 77 |

**Table 2.1.1.** Preparation for Percoll discontinuous gradients

Three layers were developed in the gradient with synaptosomes purifying as a single band in a region originally forming the 23% and 10% Percoll interface whilst the 3% and the 23% Percoll contained myelin and mitochondrial elements respectively. The

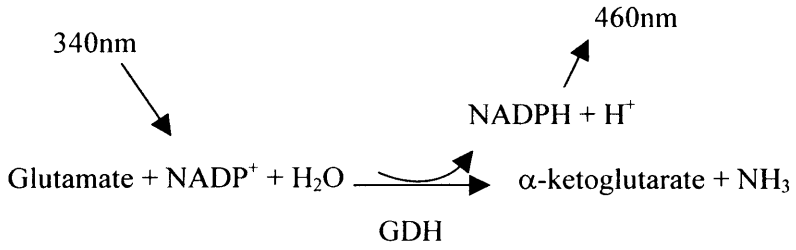
synaptosomal layer was harvested and transferred into Hepes-buffered medium (HBM) (containing:140mM NaCl, 5mM KCl, 5mM $NaHCO_3$, 1.2mM $NaH_2PO_4$, 1mM $MgCl_2$, 10mM glucose, 1mg/ml BSA and 10mM Hepes; pH 7.4) which is an experimental medium that mimicks the ionic constitution of cerebrospinal fluid. The diluted synaptosomes were then centrifuged at RCF 27,216xg for 10 min as a wash step to remove any sucrose and Percoll left in the suspension. The final synaptosomal pellet was resuspended again in 2ml of HBM using a Dounce homogeniser. Synaptosomal protein concentration was determined using the Bradford assay (Bradford, 1976) by generating a standard curve with 5, 10, 15 and 20μg bovine serum albumin (BSA) and duplicates of 5 and 10μl of the synaptosomal resuspension. The usual protein yield from one rat ranged between 1-2 mg. To obtain a specific amount of protein for experimentation, appropriate volumes of the resuspension was added to 9ml of HBM and centrifuged at RCF 3020xg at 4°C for 10 min, as a final wash. Pellets were stored on ice and generally used immediately after the preparation although synaptosomes remained viable for 6-8 hours.

**2.2. Glutamate release experiments**

Previously, a number of techniques have been used to measure glutamate release from synaptosomal preparations, including the use of isotopic labels and high performance liquid chromatography (HPLC). Isotopic detection of glutamate release was carried out with the use of exogenous [$^3$H] or [$^{14}$C]-glutamate where the radiolabel was first taken up into terminal cytosol via the plasma membrane glutamate transporter and subsequently into the synaptic vesicles by the vesicular uptake system (Nicholls and Sihra, 1986). Upon depolarisation, the radiolabelled glutamate was released by exocytosis and measured by liquid scintillation spectroscopy. However, as the specific activity of the radiolabel cannot be predicted in the vesicular pool activity and that in the cytosol activity remains high, the amount of $Ca^{2+}$-dependent vesicular/exocytotic glutamate release was often under-estimated in comparison to the cytosolic glutamate effluxed $Ca^{2+}$-independently by the reversal of the plasma membrane transporter.

Efforts to use exogenous D- [$^3$H]-aspartate as an analogue to reflect glutamate release (Potashner and Gerard, 1983) had also been proven unreliable as there is evidence suggesting that the radiolabel does not compete with glutamate for vesicular uptake (Nicholls and Attwell, 1990). This means that the radiolabel may not be effectively accumulated in vesicles, and thus, may not reflect vesicular release accurately. The use of HPLC allowed actual levels of endogenous glutamate release to be measured. However, as the technique measures output of release discontinuously, it is labour intensive and tends to require the use of a large amount of protein, it is not always the preferred method for glutamate release measurements.

Alternative to HPLC, the use of an enzyme-linked fluorometric technique has been developed for measuring glutamate release on-line and continuously (Nicholls and Sihra, 1986). The assay was based on measurement of the increase in fluorescence (excitation and emission wavelengths of 340nm and 460nm respectively) due to the production of NADPH. NADPH was produced as a result of oxidative deamination of released glutamate catalysed by the enzyme glutamate dehydrogenase (GDH) exogenously applied **(Schematic 2.2)**. Given that the reaction predicts a direct 1:1 molar relation between the NADPH produced and the glutamate released, the amount of glutamate released from the nerve terminal can be measured. As discussed in the paper by Sanchez-Prieto *et al.* (Sanchez-Prieto et al., 1987) , $NADP^+$ was utilised in this assay rather than $NAD^+$, the physiological cofactor, as it is equally effective for the reduction process, but because mammalian oxidases do not recognise the reduced NADPH, it does not undergo reoxidation which would otherwise occur with NADH.



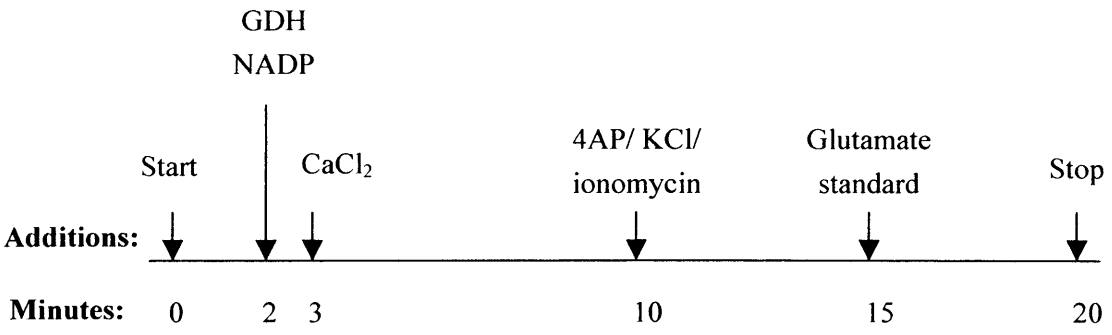

**Schematic 2.2. Experimental reaction:** glutamate is oxidised by GDH, which is coupled to $NADP^+$ reduction to cause formation of NADPH. GDH was exogenously added in large amounts in order for the enzyme to efficiently process the low levels of vesicular glutamate, with high cofactor concentration ensuring the equilibrium of the reaction tends towards the production of α-ketoglutarate and NADPH.

Although this fluorimetric technique was originally developed using synaptosomes purified from guinea-pig cerebral cortices (Nicholls and Sihra, 1986), subsequent studies have shown that this protocol is useful for studying release from other synaptosomal preparations from rat and mouse brains (Rodriguez-Moreno and Sihra, 2004; Jovanovic et al., 2000; Sanchez-Prieto et al., 1987). The fluorimetric assay has several advantages in that it is highly sensitive, being able to detect glutamate release from as little as 100μg of synaptosomal protein. Release can also be monitored continuously in real-time to obtain a single time course from one sample without the need of multiple sampling.

### 2.2.1. Protocol for glutamate release measurement by on-line fluorimetry

Synaptosomal pellets were resuspended in the HBM containing 1mg/ml of BSA and incubated in a stirred, thermostatted cuvette at 37°C in the Perkin-Elmer LS-3B spectrofluorimeter (Perkin-Elmer, Emeryville, CA). $NADP^+$ (2mM), glutamate dehydrogenase (GDH: 50 units/ml) and $CaCl_2$ (1mM) were added after 3 min. To dissect a particular pathway and examine the effect of any cross-talk with other cascades, relevant drugs were added as detailed in the results sections and figure legends. After a further 7 min, the secretagogue 4AP (1mM) was added to evoke

glutamate release. 4AP was added to induce depolarisation by acting as a $K^+$ channel blocker. KCl was used to produce depolarisation by 'clamping' the $K^+$ equilibrium




**Schematic 2.2.1.** Schematic diagram showing the times and additions during the glutamate release assay

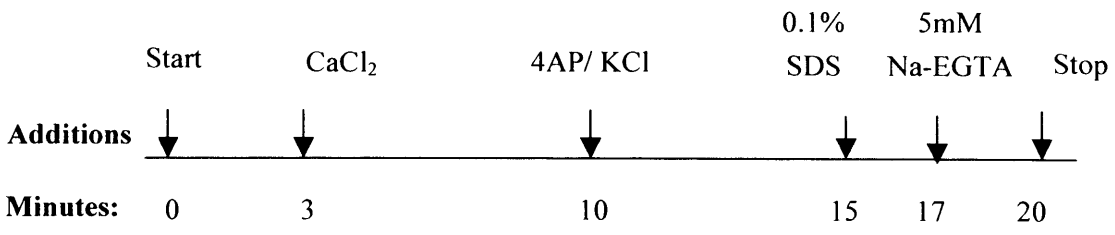
potential at depolarised levels. In both instances, depolarisation then allows $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels (VGCCs) which are generally closely juxtaposed to neurotransmitter release sites (Reid et al., 2003; Catterall, 1998; Bowman et al., 1993). Ionomycin, acting as a $Ca^{2+}$ ionophore, was used to evoke release independently of depolarisation to examine modulatory effects downstream of depolarisation-mediated $Ca^{2+}$ entry. Data were accumulated at 2-sec intervals. As an internal standard, 5nmol exogenous glutamate was added at the end of each experiment to enable the extent of glutamate release to be quantified as nmol glutamate/mg synaptosomal protein. Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin.

## 2.3. Cytosolic $Ca^{2+}$ measurements using fura-2

Cytosolic $Ca^{2+}$ measurements in synaptosomes have been made possible by the use of fura-2 (Perkinton and Sihra, 1999). Fura-2 is a ratiometric calcium indicator that fluoresces in response to binding to $Ca^{2+}$. Upon $Ca^{2+}$ binding, fura-2 exhibits a wavelength shift in the peak of the excitation spectrum from 380nm to 340nm. By calculating the ratio between these two excitation values and measuring emission of the dye at 510nm, the absolute amount of cytosolic $Ca^{2+}$ in the preparation can then be

evaluated. This ratiometric measurement is advantageous as it obviates problems associated with measuring $Ca^{2+}$ in cells of unequal thickness and differences in day-to-day dye loading, as well as leakage or photobleaching of the dye. In order to make the normally impermeable fura-2 cell-permeant, fura-2 is esterified with an acetoxymethyl (AM) group, and the lipophilic fura-2-AM loaded into cells. Once the fura-2-AM is inside the nerve terminal, the AM group is hydrolysed off by cytosolic esterases and fura is trapped intracellularly to act as a $Ca^{2+}$ indicator (Grynkiewicz et al., 1985).

Synaptosomes (0.25mg/ml) were resuspended in the HBM containing 0.1mM $CaCl_2$ and 1mg/ml BSA and loaded with 5μM fura-2-AM for 20 min at 37°C in a stirred test-tube. This preincubation with fura-2-AM was to ensure adequate incorporation of the dye into the nerve terminal to produce reliable and reproducible measurements of cytosolic $Ca^{2+}$. After fura-2-AM loading, synaptosomes were centrifuged in a microfuge for 1 min at 10,000xg. The pellets were cooled for 5 min and resuspended in HBM containing 1mg/ml BSA and the synaptosomal suspension was stirred at 37°C in a thermostatted cuvette in a Perkin-Elmer LS-3B spectrofluorimeter (Perkin-Elmer, Emeryville, CA). $CaCl_2$ (1mM) was added after 3 min and secretagogues were added at 10 min. Drugs of interest were added at appropriate times as shown in figure legends.




**Schematic 2.3.** Schematic diagram showing the times and additions during the cytosolic $Ca^{+}$ assay

Fluorescence was monitored at excitation wavelengths of 340 and 380nm and at an

emission wavelength of 505nm and data were accumulated at 3.5 sec intervals. Calibration procedures previously described (Sihra et al., 1993) were used to define the maximum and minimum fluorescence levels. SDS (0.1%) was added at 15 min to obtain the maximal fluorescence. As SDS lyses the nerve terminal, it allows the loaded fura-2 to be completely saturated in the lysate by the excess 1mM $CaCl_2$ present. On the other hand, Na-EGTA (5mM, buffered with 1M Tris) was added at 17 min to determine minimal fluorescence by acting as a $Ca^{2+}$ chelator to bind to the free $Ca^{2+}$ and, in so doing, avoid interaction with fura-2. EGTA was first buffered with 1M Tris in order to neutralise the increase in $[H^+]$ produced due to chelation of $Ca^{2+}$ by EGTA. Cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_c$, nM) was then evaluated by using the following equation described by Grynkiewicz et al. (Grynkiewicz et al., 1985).

$$[Ca^{2+}]_c = Kd \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

where: Kd = 224nM at 37°C

R = experimental fluorescence ratio value (340nm/380nm)

$R_{min}$ = minimum fluorescence ratio value, given by EGTA addition

$R_{max}$ = maximum fluorescence ratio value, given by SDS addition

$S_{f2}$ = minimum fluorescence value at 380nm

$S_{b2}$ =maximum fluorescence value at 380nm

Cumulative data were analysed using Lotus 1-2-3.

### 2.4. ERK 1&2 activation/phosphorylation assay

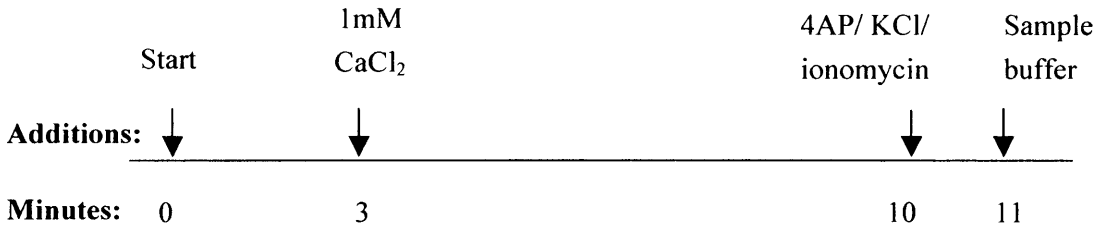
Like many kinases, the activation of ERK 1&2 can be characterised by its phosphorylation state. ERK1&2 are activated when the threonine (Thr202) and tyrosine (Tyr204) residues are dually phosphorylated by the upstream kinases, MEK

1&2. Thus, by using phospho-state specific ERK1&2 antibodies that are raised against these dually phosphorylated tyrosine/threonine residues (which has been shown to directly correspond with activation of ERK 1&2, (Payne et al., 1991)), we were able to detect the presence of active ERK1&2 in the synaptosomal preparation without the need to measure kinase activities directly. Below are descriptions of a number of incubation procedures that have been used to study different mechanisms of ERK 1&2 activation in this thesis. Note that the details and type of protocol used for specific drug incubations are further described in the methods section of each chapter.

### 2.4.1. Standard protocol for activation of ERK 1&2

ERK 1&2 activation/phosphorylation assays were performed by resuspending synaptosomal pellets in the HBM (containing 1mg/ BSA) to obtain a final protein concentration of 0.3mg/ml and incubating at 37°C. $CaCl_2$ (1mM) was added after 3 min of incubation before further addition of the drug(s) of interest as indicated in the figure legends. In experiments studying depolarisation-induced ERK activation, depolarisation was achieved by the addition of 4AP (100μM, 1mM or 3mM) or KCl (5mM, 10mM or 30mM) at 10 min before the reaction was stopped with the addition of sample buffer containing 125mM Tris (pH 6.8), 0.4% SDS(10%), 2% glycerol, 1% 3-mercaptoethanol and 0.0005% bromophenol blue at 11 min. As previous experiments ((Jovanovic et al., 2000), Davies and Sihra, personal communication) had shown that incubation at 37°C alone could produce a basal level of phosphorylation, a control incubation was always carried out in parallel at 4°C (ice). Samples were then separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and detection of ERK1&2 activation carried out by immunoblotting using phospho-state specific ERK1&2 antibodies (see section 2.4.5 and 2.4.6). Experiments were repeated with independent synaptosomal preparations three times minimally ($n \geq 3$) and the data normalised to the phosphorylation signal obtained under either 4°C control or 37°C conditions. Cumulative data were calculated as the mean ± S.E.M. of

the replicate studies.

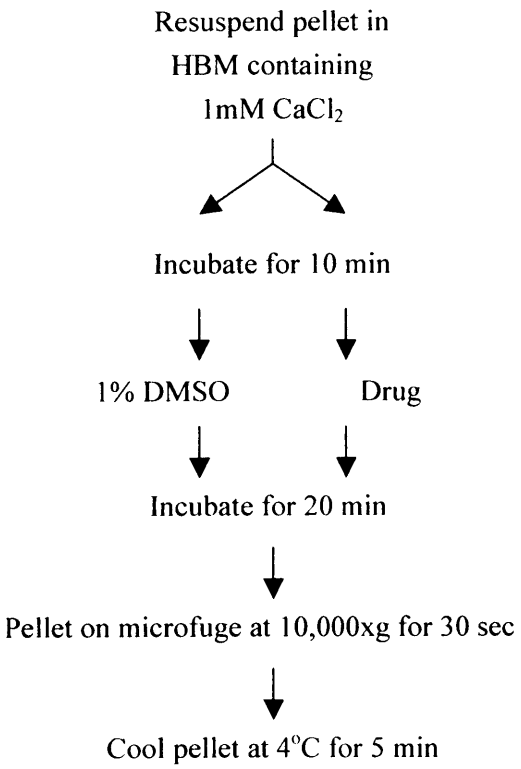



**Schematic 2.4.1.** Schematic diagram showing the times and additions during standard ERK 1&2 activation assay

### 2.4.2. Preincubation protocol

In order to identify the upstream regulators of the ERK 1&2 signalling cascade, a number of drugs have been utilised. As the permeability of the drug into the synaptosomes varies from drug to drug, in some instances, in order to facilitate the introduction of the drugs, a preincubation protocol was used prior to the standard 10-minute incubation.

Synaptosomal pellets (0.3mg/ml) were resuspended with HBM containing 1mg/ml BSA and 1mM $CaCl_2$ at 37°C in stirred test-tubes for 10 min after which time the drug of interest was added and preincubated for a further 20 min. The suspension was then pelleted in micro-centrifuge for 30 sec at 10,000xg and pellets were cooled for 5 mins at at 4°C before they were resuspended in ice-cold HBM containing 1mg/ml BSA, ready for use in the assay. The standard ERK 1&2 phosphorylation/activation assay was subsequently carried out, with the drugs of interest added at the start of the assay before transferred incubations to 37°C.

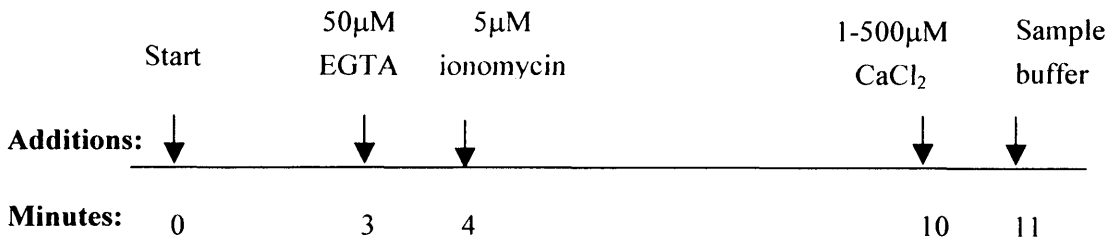



**Schematic 2.4.2.** Schematic diagram showing the times and additions during 20 min preincubation of synaptosomes

### 2.4.3. Protocol measuring $Ca^{2+}$-dependency of ERK 1&2 with ionomycin and $Ca^{2+}$-buffers

The standard protocol utilised 4AP and KCl as chemical reagents to evoke depolarisation and subsequent $Ca^{2+}$ influx via VGCCs into the nerve terminal. However, in order to study directly the effect of $Ca^{2+}$ on ERK 1&2 activation, the $Ca^{2+}$ ionophore ionomycin was used to directly titrate $Ca^{2+}$ levels in the cytosol. Thus, in the presence of EGTA (for the removal of basal/residual calcium contaminating the synaptosomal preparation and to buffer [$Ca^{2+}$]) and ionomycin, $Ca^{2+}$-dependent ERK 1&2 activation was stimulated with an exogenously added concentrations of free calcium ranging from 1-500μM.

Synaptosomal pellets (0.3mg/ml) were resuspended in the HBM containing 1mg/ml BSA and incubated at 37°C in a stirred test-tube. At 3 min, 50μM EGTA was added, to buffer both the extra- and intrasynaptosomal calcium present in the synaptosomes that may be liberated by the addition of 5μM ionomycin (added at 4 min). Varying concentrations of free calcium (0, 1, 10, 50, 100 or 500μM) were then added at 10 min to study the effect of $Ca^{2+}$ on ERK 1&2 activation. Drugs of interest were added as indicated in the figure legends. Reactions were stopped at 11 min with the addition of sample buffer and samples were subjected to SDS-PAGE and immunoblotting as described below.




**Schematic 2.4.3.** Schematic diagram showing the times and additions during $Ca^{2+}$-dependency assay

### 2.4.4. BAPTA-AM/EGTA assay protocol

The BAPTA-AM/EGTA assay was established to study the roles of intrasynaptosomal and extrasynaptosomal $Ca^{2+}$ on the activation of ERK 1&2. While EGTA was used as a $Ca^{2+}$ chelator to bind extrasynaptosomal $Ca^{2+}$, BAPTA was used in its AM-form to allow loading into the synaptosomes and subsequent hydrolysis for chelating $Ca^{2+}$ intrasynaptosomally.

Synaptosomal pellets (0.3mg/ml) were resuspended in HBM buffer containing 1mM $CaCl_2$ and then split into two test-tubes and incubated with stirring at 37°C for 10 min. DMSO (1%, control) or BAPTA-AM (100μM) was added to each tube and preincubated for a further 20 min. The suspension was then centrifuged in a

microfuge for 30 sec at 10,000xg and supernatants removed. Pellets were then cooled at 4°C for 5 min. Synaptosomal pellets were resuspended and incubated normally in stirred test-tubes with the addition of 1mM $CaCl_2$ or 100μM EGTA at 3 mins and secretagogues (4AP) at 10 min. Drugs of interest were added as indicated in the figure legends. Samples were stopped with sample buffer at 11 min and were subjected to SDS-PAGE, protein electrotransfer and immunoblotting as described in more detail below.

### 2.4.5. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) is an electrophoretic method for separating proteins according to their molecular weights. SDS is a detergent that denatures the secondary/tertiary structures of the protein. By virtue of its amphiphatic properties, SDS binds to the protein by its hydrophobic moiety with the anionic sulphate groups (negative charges) exposed. Thus, the globularised charged protein can then be separated under the electric field with SDS-PAGE by size, making use of the matrix formed by the polyacrylamides (acrylamide and cross-linker bisacrylamide). Here, a discontinuous gel system was used with gels consisting of a stacking dimension and a resolving dimension. Percentages of these gels were made up as follows to provide a high resolution and fine separation of ERK1&2. The stacking gel contained 3.75% acrylamide, 0.1% bis-acrylamide, 0.1% SDS and 125mM tris-HCl (pH 6.8) and were polymerised using 1μl/ml TEMED and 5μl/ml 10% APS. The resolving gel contained 7.5% acrylamide, 0.2% bisacrylamide, 0.1% SDS and 325mM tris-HCl (pH 8.8) and were polymerised using 0.5μl/ml of TEMED and 2.5μl/ml of 10% APS.

After polymerisation, gels were loaded with 20μg of synaptosomal protein (determined using the Bradford assay) and run at constant voltage (80V through the stacking gel and 150V through the resolving gel) at room temperature in running

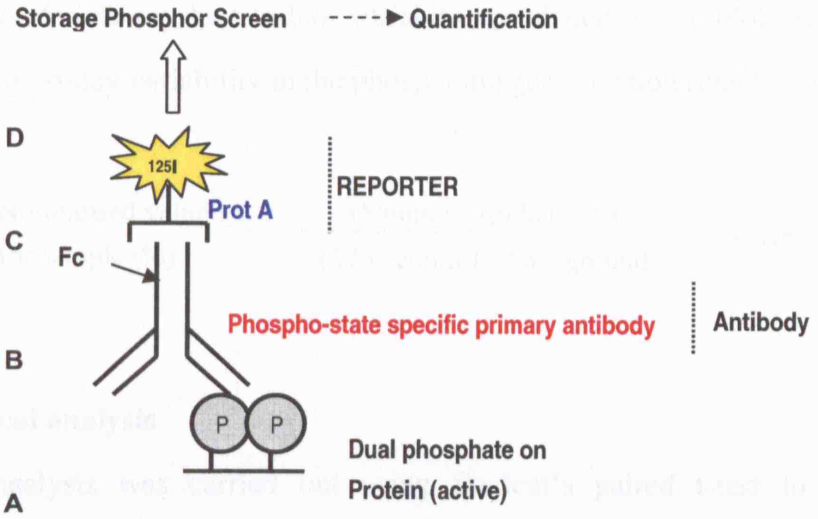
buffer consisting of 25mM Tris, 192mM glycine and 0.1% SDS (pH 8.6). Molecular weight markers (Rainbow Markers, Amersham) ranging between 14.3kDa and 220kDa were used to visibly label the gel during the electrophoresis so that positions of ERK1&2 migration could be identified.

### 2.4.6. Immunoblotting with phospho-ERK 1&2 (Thr202/Tyr204) antibody

Following SDS-PAGE, proteins were electrotransferred onto nitrocellulose (Protran) membranes, which are established vehicles for immunodetection, in electrotransfer buffer containing 23mM Tris, 192mM glycine, 20% methanol for 24 hours at constant current (200mA) and room temperature. After electrotransfer, membranes were air-dried and labelled with Ponceau S (0.2% Ponceau Red, 3% TCA and 3% sulfosalicyclic acid) to visualise whether transfer was successful and whether protein was loaded and transferred evenly. Membranes were then washed with three 10 min incubations of TBS-Tween (20mM tris, pH 7.6, 137mM NaCl, 0.1% Tween-20) to remove traces of Ponceau S. Next, they were incubated for 1 hour at room temperature with blocking buffer consisting of TBS (20mM tris, pH 7.6, 137mM NaCl), 0.1% Tween-20 and 5% nonfat dry milk to reduce non-specific binding of antibody and other contaminants to the membrane. Membranes were then subjected to immunoblotting by incubating with rabbit polyclonal phospho-p44/p42 ERK1&2 antibody (raised against dually phosphorylated ERK 1&2 at Thr202 and Tyr204) at 1:1000 dilution in TBS-Tween overnight at 4°C. This allows the detection of dually phosphorylated tyrosine and threonine residues on activated ERK 1&2.

After the overnight incubation, the membrane was washed 3 times (15 min each) with blocking buffer to remove any excess, unbound primary antibody. To report the binding of phospho-ERK 1&2 antibody to the activated ERK 1&2 on the membrane, membranes were then labelled with $^{125}$I-Protein A (Amersham Biosciences) diluted in blocking buffer for 1 hour and washed extensively (one 5 min and one 10 min with blocking buffer, followed by one 5 min and one 10 min wash with TBS-Tween-20) to

remove excess reporter label. The labelled membranes were then air-dried and exposed to a phosphoimager screen (Kodak) overnight. Immunopositive bands were visualised with a Typhoon 9410 phosphor-imager scanning system (Amersham Biosciences). **Schematic 2.4.6** shows that the membrane was radiolabelled with $^{125}$I-Protein A which specifically binds to the Fc portion of the primary antibody, thus forming a complex with dually phosphorylated ERK 1&2 on the membrane.




**Schematic 2.4.6. Immuno-quantification using phospho-specific antibodies and reporter label.** A) Protein was dually phosphorylated upon activation; B) Dually phosphorylated protein was detected by phospho-state specific antibody; C) $^{125}$I-Protein A labelled the Fc portion of antibody bound to the protein; D) Immunoreactive bands were visualised and quantified by the amount of radioactivity detected on the storage phosphor screen.

The use of $^{125}$I-Protein A have several advantages over conventional use of secondary antibodies and enhanced chemiluminescence (ECL) techniques. Firstly, binding of $^{125}$I-Protein A to the Fc portion of the primary antibody is stiochiometric, making the evaluation of signal detected easier to quantify. Secondly, the sensitivity of the reporter $^{125}$I-Protein A detection is high given that screens can be exposed for increasing periods of times. Thirdly, the half-life of the radiolabel means that, if necessary, blots can be re-evaluated over a long period of time.

### 2.4.7. Immunoblot analysis

Following the scanning of radioactive western blots using the Typhoon 9410 phosphoimager, phosphor intensities were measured and analysed using the ImageQuant software (Molecular Dynamics) where bands were highlighted with uniform-sized boxes. The same sized box was also used to sample the levels of background radioactivity and this level was later subtracted from the sample values. Values were then normalised to the 37°C control as shown below. Normalised values were used to obviate any blot-to-blot variability produced by the blotting procedures as well as day-to-day variability in the phosphoimager detection sensitivity.

$$\text{Normalised value for sample (\%)} = \frac{(\text{Sample} - \text{background})}{(37^{\circ}\text{C control} - \text{background})} \times 100$$

### 2.5. Statistical analysis

Statistical analysis was carried out using Student's paired t-test to assess the confidence levels of significant differences between two sets of data. Confidence levels of differences between multiple sets of data were examined using analysis of variance (ANOVA), followed by Dunnetts post hoc test.

## 2.6. Reagents List

Sucrose, HPLC graded water, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), tris(hydroxymethyl)methylamine (tris), glycine, sodium dodecyl sulphate (SDS) and Protran nitrocellulose membranes were all obtained from VWR (U.K.). Percoll, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), glutamate dehydrogenase (GDH), nicotinamide adenine dinucleotide (NADP), L-glutamic acid, 4-aminopyridine (4AP), potassium chloride (KCl), bovine serum albumin (BSA), ethylene glycol-bis(b-aminoehyl ether)-N,N,N',N'-tetraacetic acid (EGTA), LY29004, baclofen and GABase were obtained from Sigma-Aldrich (Dorset, U.K.). 1,2-bis-(o-Aminophenooxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM), ionomycin, lovastatin, okadaic acid, FK506, thapsigargin, KN93, roscovitine, PP2 and PP3 were purchased from Calbiochem (U.K.). Fura-2 acetoxymethyl ester (Fura-2-AM) was from Molecular Probes (Cambridge, U.K.). Acrylamide and bis-acrylamide were purchased as ready-made solutions (30% and 2% solutions, respectively) from National Diagnostic (U.K.). High range Rainbow markers and $^{125}$I-labelled Protein A were obtained from Amersham Bioscience (U.K.). Rabbit polyclonal phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibody (phospho-ERK1,2 antibody) was from New England Biolabs (Hertfordshire, U.K.). W7 was obtained from Tocris. BDNF was a gift from Regeneron Pharmaceuticals. PD98059 was a gift from A.R. Saltiel (Parke-Davis Pharmaceutical Research Division of Warner-Lambert). SNX-482 was a gift from Alomone Laboratory.

# Chapter 3

# UPSTREAM AND DOWNSTREAM REGULATORS OF ERK 1 & 2

## 3.1. Introduction

The ability of neurons to undergo activity-dependent changes in synaptic strength forms an important basis for the functional connectivity underlying learning and memory. Such plasticity, occurring to either enhance or reduce signal transmission across the synapses, can lead to short-term or long-lasting alterations. Over the years, numerous signalling systems have been implicated as playing crucial parts in regulating this synaptic plasticity. Among these, the role of extracellular signal-regulated kinases (ERK) 1&2 signalling pathway has been increasingly studied.

The view that ERK 1&2 are essential components of synaptic transmission and plasticity came from studies in which the inhibitor of MEK, PD98059, efficiently blocked NMDA-dependent long-term potentiation (LTP), indicating that ERK 1&2 activation is necessary for the induction of this type of plasticity (Watanabe et al., 2002; Watabe et al., 2000; Winder et al., 1999). Various findings also suggested the involvement of ERK 1&2 signalling in other forms of plasticity, including NMDA receptor-independent neurotransmission in the hippocampal CA1 region (Kanterewicz et al., 2000), and LTP in the dentate gyrus (Coogan et al., 1999), cortex (Di Cristo et al., 2001) and amygdala (Huang et al., 2000; Schafe et al., 2000). Indeed, the disruption of this ERK 1&2-mediated LTP at the Schaffer collateral synapses has been shown to lead to neurofibromatosis, a learning disorder associated with mental retardation and hippocampus-dependent memory deficits (Costa et al., 2002). In addition, studies have associated a role for ERK 1&2 in AMPA receptors insertion (Zhu et al., 2002) and dendritic spine formation and stabilisation (Thomas and Huganir, 2004; Wu et al., 2001b).

Together with evidence supporting the involvement of ERK 1&2 in postsynaptic plasticity, increasingly, studies have shown that ERK 1&2 can participate in the modulation of presynaptic plasticity by regulating mechanisms underlying

neurotransmitter release. ERK 1&2 have been implicated to play a major role in the regulation of the phosphorylation state of synapsin, a family of presynaptic phosphoprotein involved in the modulation of synaptic vesicle (SV) availability (Chi et al., 2003; Yamagata et al., 2002; Jovanovic et al., 2001; Jovanovic et al., 1996). In *Aplysia*, modulation of presynaptic functions in neurons has been shown to be mediated by the activation of ERK 1&2 (Sharma et al., 2003; Martin et al., 1997) and this may be through ERK 1&2-dependent phosphorylation of synapsin I (Chin et al., 2002). Studies in rat cerebrocortical synaptosomes also indicated a concomitant facilitation of glutamate release when phosphorylation of synapsin at phosphosites 4,5 by ERK 1&2 was stimulated by the neurotrophin BDNF (Jovanovic et al., 2000). Given that acute treatment of BDNF leads to a reduced ability of synapsin to bundle actin and to promote G-actin polymerisation (Jovanovic et al., 1996), these data present a presynaptic mechanism by which ERK 1&2 activation can modulate the function of synapsin following neuronal stimulation.

Consistent with a presynaptic role of ERK 1&2 signalling, H-Ras, a small GTP-binding protein and a known ERK signalling regulator, has been shown to result in an increased frequency of mEPSCs, the facilitation of neurotransmitter release as well as the consequent increases in LTP in hippocampal neurons (Kushner et al., 2005). Indeed, this Ras-dependent ERK 1&2 signalling has been established in many systems as a "classic" pathway for neurotrophin-mediated ERK 1&2 activation (Wellbrock et al., 2004; Bos, 1998). In this pathway, Ras is activated by guanine nucleotide exchange factors (GEFs) following receptor tyrosine kinase activation, where phosphotyrosines serve as docking sites for the assembly of the adaptor protein Grb2 and GEF Sos. Activated Ras then allows the subsequent activation of Raf-1 proteins to relay signals to the MAPK/ERK kinases (MEKs), which act to phosphorylate, and thus, activate ERK 1&2. The mechanism of ERK 1&2 activation is, however, complicated by the presence of another pathway involving Rap1 and its effector, B-Raf (Vossler et al., 1997; Kitayama et al., 1989). Studies have

demonstrated that Rap1 and B-Raf are the important regulators in the sustained function of ERK 1&2 as opposed to Ras and Raf-1, which are thought to involved only in the transient activation of ERK 1&2 (York et al., 1998). However, with other data showing both antagonising and potentiating effect of the two signalling cascades (Stork, 2003; Bos et al., 2003; Bos et al., 2001; Zwartkruis and Bos, 1999), questions are being asked about the physiological relevance of the Ras/Raf-1 and the Rap1/B-Raf mediated ERK 1&2 pathways. Clearly, further work is required to disclose the precise mechanisms by which ERK 1&2 is activated following stimulation.

In addition to the upstream regulation of ERK 1&2 activation by the small GTP-binding proteins and the upstream protein kinases, the activities of ERK 1&2 can also be, in part, modulated by the presence of specific protein phosphatases. A family of MAPK phosphatases, known as MKP1, 2 and 3, has been suggested to provide temporal controls of the activities of ERK 1&2 by mediating dephosphorylation of ERK 1&2 on their phosphothreonine and phosphotyrosine residues (Camps et al., 2000; Keyse, 2000). *In vitro* studies showed that a negative feedback loop to ERK 1&2 could occur involving these MKPs (Brondello et al., 1999; Brondello et al., 1997), although the precise mechanisms of activation of these MKPs are still unclear.

Besides regulation by MKPs, a large amount of evidence has suggested that ERK 1&2-mediated changes in synaptic plasticity may be modulated by the serine/threonine protein phosphatases. In this respect, protein phosphatase 1 (PP1) has been implicated in the induction of synaptic plasticity such as LTD (Winder and Sweatt, 2001), where PP1 has been shown to dephosphorylate GluR1 as well as membrane-bound CaMKIIα (Lee et al., 2000a; Strack et al., 1997a). In addition, there is evidence that ERK 2 phosphorylation was sensitive to okadaic acid, a general PP1 and PP2A inhibitor, in the hippocampus, suggesting that PP1 may regulate the activity of ERK to subsequently contribute to the modulation of synaptic function (Runden et al., 1998).

In both neuronal and non-neuronal preparations, studies have also highlighted the function of protein phosphatase 2A (PP2A) in downregulating presynaptic ERK-regulation of synaptic transmission (Jovanovic et al., 2001; Alessi et al., 1995). This has been supported by experiments using okadaic acid, which demonstrated the ability of PP2A, but not PP1, to dephosphorylate synapsin at phosphosites 1,2,3 (Jovanovic et al., 2001). In contrast to this, however, there is evidence that PP2A positively regulates Ras-ERK 1&2 signalling by dephosphorylating the kinase suppressor of Ras (KSR), which is normally active in its phosphorylated form. In other studies, PP2A has also been involved in reversing the hyperphosphorylated /desensitised form of Raf-1, and thus, allow for its return to signalling-competent state (Dougherty et al., 2005; Ory et al., 2003). Given the bidirectionality of the potential effect of PP2A on Ras/ERK signalling, the precise function of PP2A in the regulation of ERK 1&2 further warrants investigation.

The $Ca^{2+}$-dependent phosphatase, protein phosphatase 2B (PP2B) or calcineurin, has been implicated in the regulation of glutamate release by inhibiting release at the level of VGCC $Ca^{2+}$ influx (Burley and Sihra, 2000; Sihra et al., 1995). There is also evidence to suggest that calcineurin is involved in the modulation of SV trafficking by mediating $Ca^{2+}$-dependent dephosphorylation of synapsin at phosphosites 4,5,6 (Chi et al., 2003; Jovanovic et al., 2001). In addition, calcineurin has been shown to inhibit Ras/Raf/MAPK signalling following NGF stimulation in neuroblastoma cells (Price et al., 2003). However, the role of calcineurin in directly coordinating the activities of ERK 1&2 in the synaptic terminal has not been determined.

This chapter aims to examine the details of ERK 1&2 activation underlying the release of glutamate in the presynaptic nerve terminal. To this end, direct phosphorylation of ERK 1&2 in rat cerebrocortical synaptosomes was studied. In particular, the mechanism of neurotrophin-mediated activation of ERK 1&2 was

investigated to ask whether ERK 1&2 activation in synaptosomes follows the "classic" signalling pathway involving the activation of upstream regulator Ras, Raf and MEK. Finally, the potential regulation of ERK 1&2 activities by protein phosphatases PP1, PP2A and calcineurin was explored.

## 3.2. Materials and Methods

### 3.2.1. Synaptosomal preparation

Synaptosomes were prepared as described in section 2.1.1.

### 3.2.2. Glutamate release experiment

Glutamate release was monitored by on-line fluorimetry as described in section 2.2.1. Synaptosomes were resuspended in HBM containing 1mg/ml BSA, after which they were incubated in the presence of 50U/ml GDH and 2mM $NADP^+$, with BDNF (200ng/ml) or lovastatin (10μM, with preincubation) added at the start of the incubation. $CaCl_2$ (1mM) was then added at 3 min and depolarisation was achieved with the addition of 4AP (1mM) at 10 min.

### 3.2.3. Standard incubation protocol for ERK 1&2 activation/phosphorylation

The 10-minute standard incubation protocol was followed as described in section 2.4.1. Synaptosomes were resuspended in HBM containing 1mg/ml BSA. They were then incubated at 37°C with BDNF (200ng/ml), PD98059 (50μM), lovastatin (10μM), okadaic acid (1, 10, 100nM) or FK506 (1μM), added at the start of the incubation. $CaCl_2$ (1mM) was added at 3 min before secretagogue addition at 10 min. The reactions were then terminated with the addition of sample buffer 1 min after the secretagogue.

### 3.2.4. Preincubation protocol

Synaptosomes were preincubated at 37°C in HBM containing 1mg/ml BSA and 1mM $CaCl_2$. After 10 min, vehicle/DMSO (1%) or lovastatin (10μM) was added and synaptosomes were further incubated for 20 minutes. Synaptosomes were then pelleted at 10,000xg using a bench-top microcentrifuge. The supernatants were discarded and the pellets cooled back to 4°C for 5 min before following the standard

incubation protocol.

### 3.2.5. Protocol measuring $Ca^{2+}$-dependency of ERK1&2 with ionomycin and $Ca^{2+}$-buffers

Synaptosomes were incubated for 10 minutes in the HBM buffer containing 1mg/ml BSA for 10 minutes as detailed in section 2.4.3. EGTA (50μM) as $Ca^{2+}$-buffer was added at 3 min followed by the addition of 5μM ionomycin at 4 min. Calcium (500μM buffered-free $CaCl_2$) was then added at 10 min before the reactions were stopped with the addition of sample buffer. Drugs of interest were added as described in the figure legends.

### 3.2.6. SDS-PAGE and Immunoblotting

Samples were separated on SDS-PAGE and subjected to immunoblotting as described in detail in sections 2.4.5 and 2.4.6.

### 3.2.7. Statistical analysis

Student's paired t-test was carried out to assess the confidence levels of significant differences between two sets of data.

### 3.2.8. Reagents

A stock solution of 1mg/ml BDNF (Brain-derived neurotrophic factor) was made in buffer containing PBS before diluting to working solution of 20μg/ml in water. BDNF was used at final concentration of 200ng/ml.

A stock solution of 10mM lovastatin was made using DMSO before diluting to a 1mM working solution in HBM/BSA. Lovastatin was used at 10μM as a final concentration.

PD98059 stock solution was made at 50mM using DMSO. This was then diluted further to 5mM with HBM/BSA for used as the working solution. PD98059 was used at a final concentration of 50μM.

Okadaic acid stock solution of 100mM was made using DMSO and working solutions of 100nM, 1μM and 10μM were obtained by further dilutions using HBM/BSA. Okadaic acid was used at various concentrations of 1, 10 and 100nM.

A 10mM stock solution of FK506 was made using DMSO, which was further diluted to 100μM with HBM/BSA prior to use. FK506 was used at final concentration of 1μM.

### 3.3. Results

In order to examine the presynaptic action of BDNF, nerve terminals (synaptosomes) from rat cerebral cortices were isolated and depolarisation of the nerve terminal was achieved using 4-aminopyridine (4AP) (Fig. 3.1A). 4AP acts as a $K^+$ channel blocker to block outward $K_A$ currents at the plasma membrane and, in so doing, destabilises the resting plasma potential to increase the probability of voltage-gated $Na^+$ channels (VGSC) firing and thus depolarises the synaptosomes. On-line fluorometric measurement of glutamate release from the preparation revealed that the addition of 1mM 4AP could evoke the release of about 25 nmol glutamate/mg synaptosomal protein after 240 sec of depolarisation (Fig. 3.1B). Preincubation of synaptosomes with 200ng/ml BDNF resulted in a potentiation of release to 33 nmol/mg. As BDNF has been established as a positive modulator of ERK 1&2, this enhancement of glutamate release of 8 nmol/mg confirms the previously reported facilitation of BDNF-induced glutamate release occurring through the activation of ERK 1&2 following TrkB activation (Jovanovic et al., 2000).

Next, we probed the activation of ERK 1&2 in synaptosomes with antibody raised against the dually phosphorylated or active form of ERK. Immunoblot analysis showed that both isoforms of ERK, ERK 1 and ERK 2, were phosphorylated in the nerve terminal at 4°C (Figure 3.2A). The upper band represents ERK 1, running at the MW of 44kDa and the lower band represents p42, the ERK 2 isoform. Under conditions where the temperature was raised to physiological levels of 37°C, the activation of ERK 1&2 was substantially potentiated so that ERK 1&2 phosphorylation was 92.8 ± 9.3 % greater compared to the 4°C control (Figure 3.2B). Note that, for quantification purposes, ERK 1&2 bands were combined so that the total ERK 1&2 activation levels were evaluated. The finding that ERK 1&2 activation was stimulated at 37°C in HBM without any exogenous stimulation suggests that a high level of basal ERK 1&2 activation is present in synaptosomes under "resting"

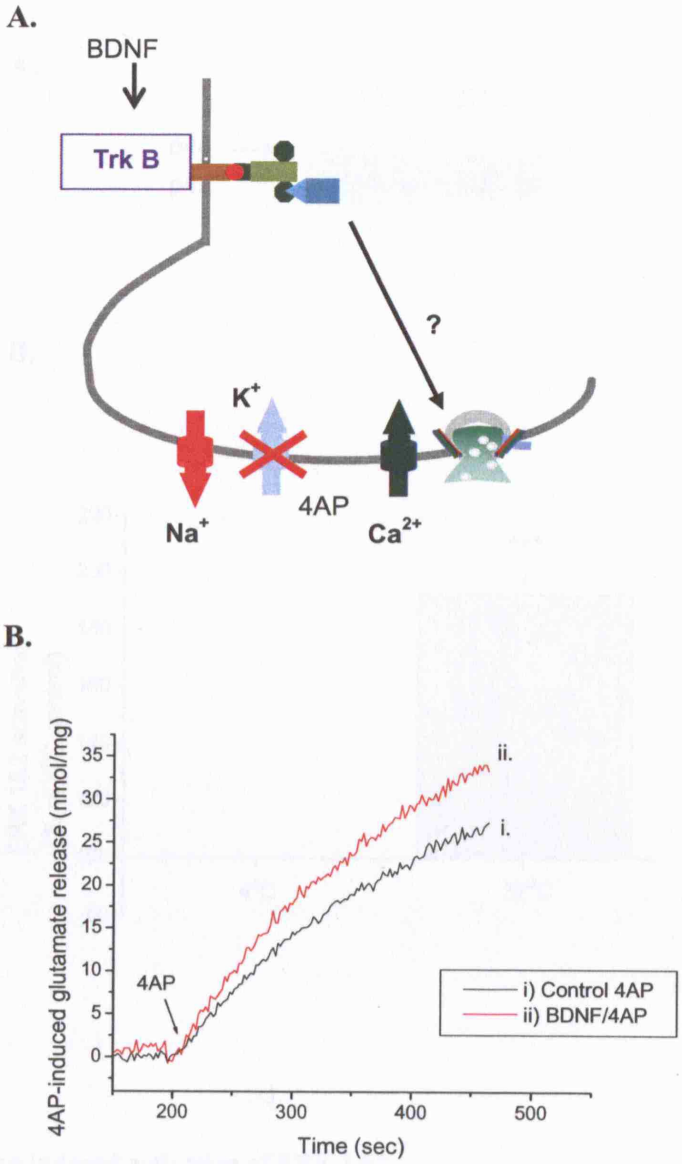



**Figure 3.1. Effect of neurotrophin BDNF on 4AP-evoked glutamate release.**

**A.** Scheme showing proposing effect of BDNF on depolarisation-induced glutamate release. **B.** Glutamate release: i) Control 4AP; ii) BDNF/4AP. Synaptosomes (0.1 mg/ml) were incubated in the presence of 1mM of $CaCl_2$ and release was followed by on-line fluorimetry under standard conditions as indicated in the Materials and Methods section. Glutamate release was elicited with the addition of 1mM 4AP in the absence (i) or presence (ii) of 200ng/ml BDNF added at the start of the experiment. The experiment was carried out with one synaptosomal preparation (n=1).

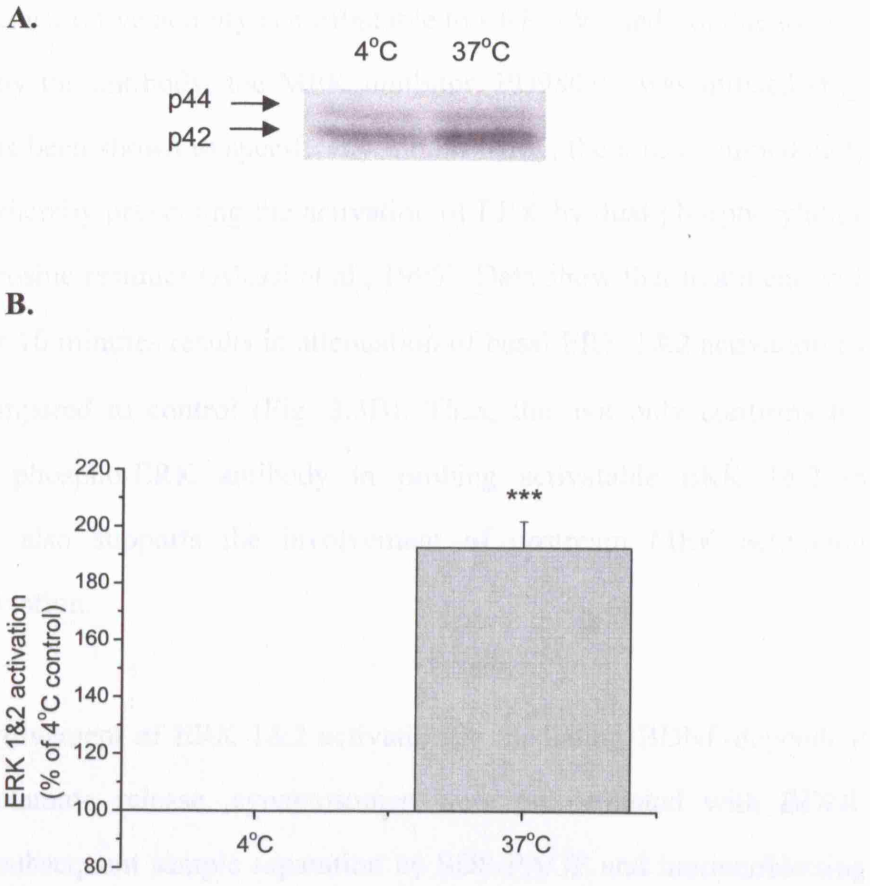



**Figure 3.2. Temperature-induced activation of ERK 1&2.**

**A.** Phosphoimages of phospho-ERK in 4°C and 37°C-incubated synaptosomal preparation. **B.** Quantification of phospho-ERK 1&2 levels. Synaptosomes (0.3 mg/ml) were incubated for 10 min at 4°C or 37°C with 1mM of $CaCl_2$ added at 3 min. Samples were then separated by SDS-PAGE and transferred onto nitrocellulose membrane as described in the Materials and Methods section. Blots were treated with phospho-p44/42 MAPK (Thr202/Tyr204) antibody (NEB) at 1:1000 dilution overnight at 4°C and probed using $^{125}$I-labelled Protein A. Immuno-bands were detected and analysed with a Typhoon phosphoimager (Molecular Dynamics). Data are plotted as % of 4°C control of ERK 1&2 activation. ***$p<0.001$ compared to 100% 4°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with six independent synaptosomal preparations (n=6).

physiological conditions.

To verify that this constitutive activity is attributable to ERK 1&2 and not due to non-specific detection by the antibody, the MEK inhibitor, PD98059, was utilised (Fig. 3.3A). PD98059 has been shown to specifically inhibit MEK, the kinase immediately upstream of ERK, thereby preventing the activation of ERK by dual phosphorylation at threonine and tyrosine residues (Alessi et al., 1995). Data show that treatment with 50μM PD98059 for 10 minutes results in attenuation of basal ERK 1&2 activation by 40.8 ± 10.2 % compared to control (Fig. 3.3B). Thus, this not only confirms the specificity of the phospho-ERK antibody in probing activatable ERK 1&2 in synaptosomes, but also supports the involvement of upstream MEK activation preceding ERK activation.

To validate the involvement of ERK 1&2 activation in mediating BDNF-dependent potentiation of glutamate release, synaptosomes were preincubated with BDNF (200ng/ml) before subsequent sample separation by SDS-PAGE and immunoblotting with the phospho-specific ERK antibody (Fig. 3.4A). Results demonstrate that the addition of BDNF positively modulates the activities of ERK 1&2, with preincubation of BDNF (200ng/ml) leading to a potentiation of basal ERK 1&2 activation to 20.4 ± 9.0 % above control level (Fig. 3.4B).

Having established ERK 1&2 activation in BDNF-dependent modulation of synaptic release, the next experiment aimed to evaluate the upstream signalling pathway leading to the activation of ERK following Trk activation. While various studies have demonstrated the involvement of the small GTPase binding protein Ras in the "classic" pathway of ERK 1&2 activation, this has not been demonstrated directly with the presynaptic ERK pathway examined herein. We directly address the function of Ras in the BDNF-mediated ERK 1&2 signalling cascade by using a specific Ras

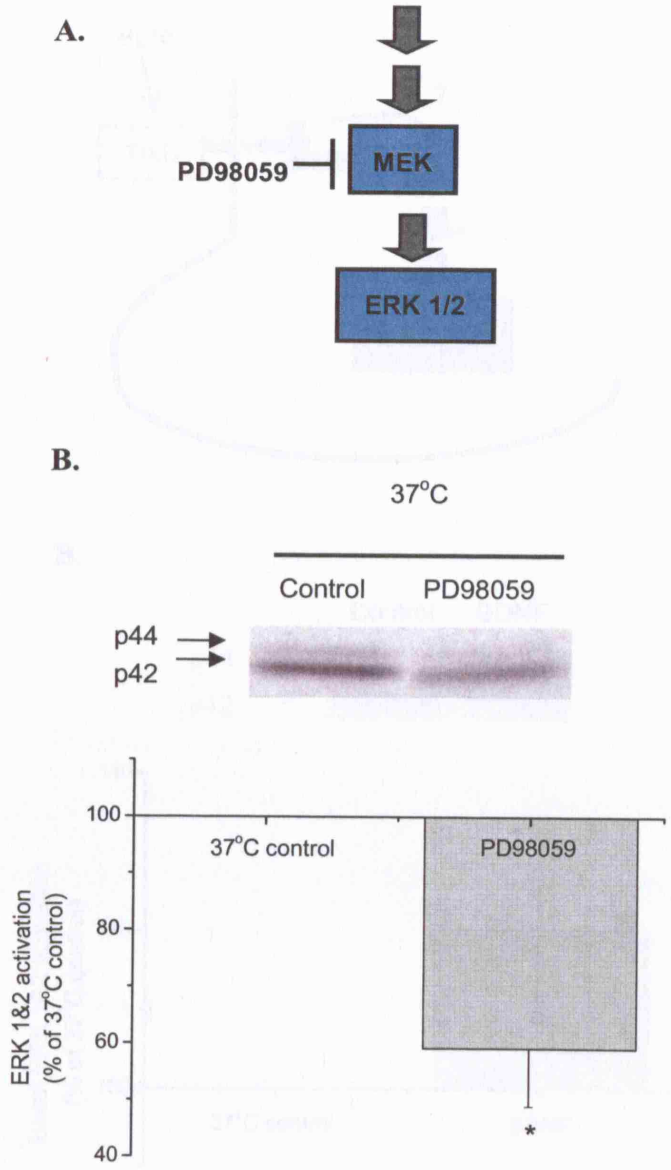



**Figure 3.3. Effect of PD98059, a MEK inhibitor, on temperature-induced ERK 1&2 activation.**

**A.** Proposed effect of PD98059 on basal ERK 1&2 levels at 37°C. **B.** Phosphoimages and quantification of phospho-ERK in basal synaptosomal preparation with and without treatment of PD98059. Synaptosomes (0.3 mg/ml) were incubated in the absence and presence of MEK inhibitor, PD98059 (50μM). Protein separation by SDS-PAGE and immunoblotting were carried as described in the Materials and Methods section. Data are plotted as % of 37°C/temperature-induced control of ERK 1&2 activation. *$p<0.05$ compared to 100% 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with three independent synaptosomal preparations (n=3).

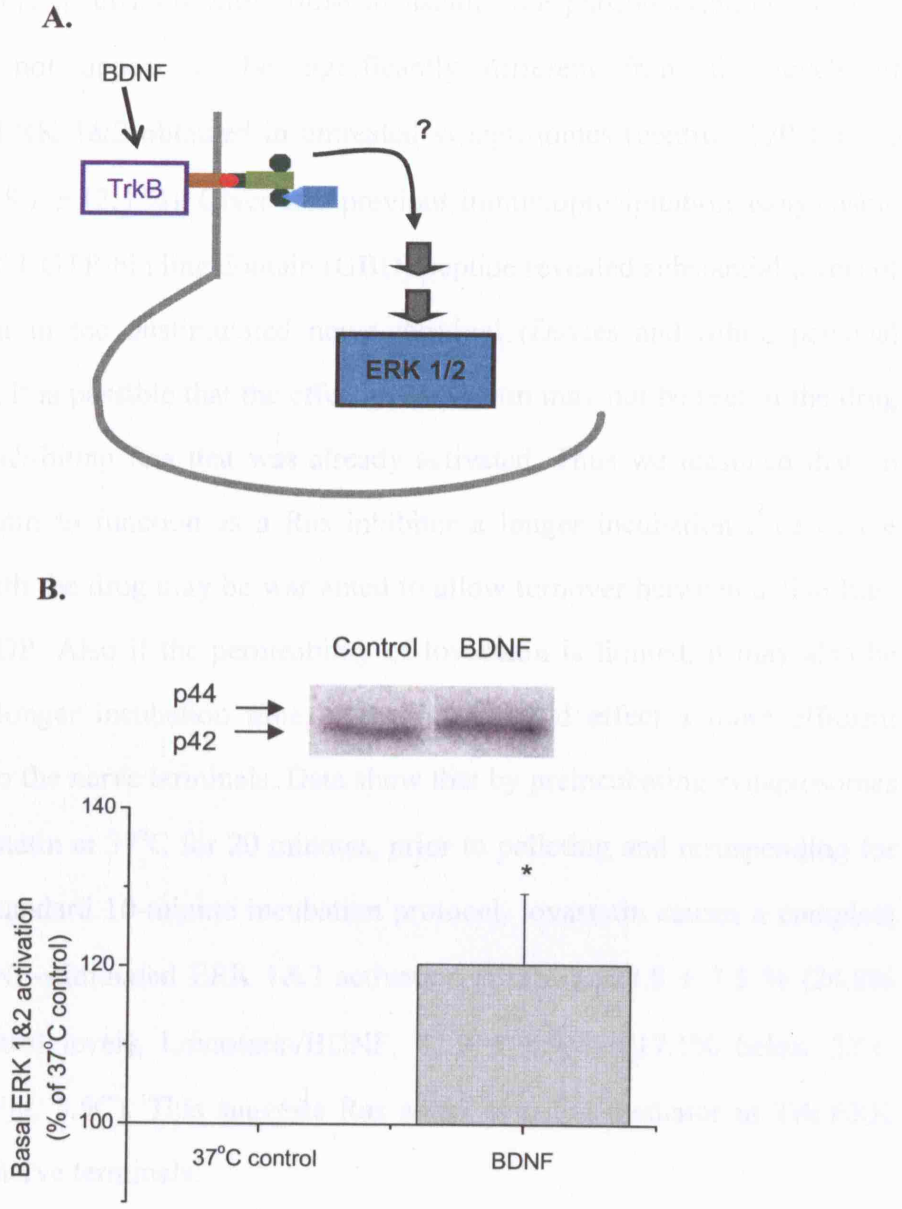



**Figure 3.4. BDNF potentiates basal ERK 1&2 activation.**

**A.** Scheme showing proposed effect of BDNF on basal ERK 1&2 levels at 37°C. **B.** Phosphoimages and quantification of effect of BDNF on phospho-ERK in basal synaptosomal preparation. Synaptosomes (0.3 mg/ml) were incubated with or without 200ng/ml BDNF. Protein separation by SDS-PAGE and immunoblotting were carried as described in the Materials and Methods section. *$p<0.05$ compared to 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with five independent synaptosomal preparations (n=5).

inhibitor, lovastatin (Reusch et al., 1995) (Fig. 3.5A). Figure 3.5B shows that upon standard 10-minute incubation with 10μM lovastatin, the phosphorylation levels of ERK 1&2 do not appear to be significantly different from the levels of phosphorylated ERK 1&2 obtained in untreated synaptosomes (control, 120.4 ± 9.0 %; lovastatin, 118.1 ± 12.3 %). Given that previous immunoprecipitation assays using antibodies to Raf-1 GTP-binding domain (GBD) peptide revealed substantial levels of Ras-GTP present in the unstimulated nerve terminal (Davies and Sihra, personal communication), it is possible that the effect of lovastatin may not be seen if the drug is incapable of inhibiting Ras that was already activated. Thus we reasoned that, in order for lovastatin to function as a Ras inhibitor a longer incubation time of the synaptosomes with the drug may be warranted to allow turnover between active Ras-GTP and Ras-GDP. Also if the permeability of lovastatin is limited, it may also be possible that a longer incubation time of the drug would effect a more efficient incorporation into the nerve terminals. Data show that by preincubating synaptosomes with 10μM lovastatin at 37°C for 20 minutes, prior to pelleting and resuspending for the subsequent standard 10-minute incubation protocol, lovastatin causes a complete abolition of BDNF-stimulated ERK 1&2 activation (BDNF, 124.8 ± 7.5 % [24.8% above 37°C control level]; Lovastatin/BDNF, 82.9 ± 8.9 % [17.1% below 37°C control level]) (Fig. 3.5C). This suggests Ras as an essential mediator in Trk-ERK signalling in the nerve terminals.

To examine the involvement of upstream Ras activation underlying release, glutamate release was monitored in the presence of the Ras inhibitor lovastatin (Fig. 3.6A). Following 20 minutes preincubation with 10μM lovastatin, 4AP-evoked glutamate release was attenuated to 26.0 ± 0.6 nmol/mg compared to a control release of 34.5 ± 1.5 nmol/mg (at 240 sec after depolarisation) (Fig. 3.6B). This clearly indicates the role of Ras in modulating mechanisms underlying neurotransmitter release and hence suggests the presence of the "classic" Ras-mediated ERK 1&2 cascade in

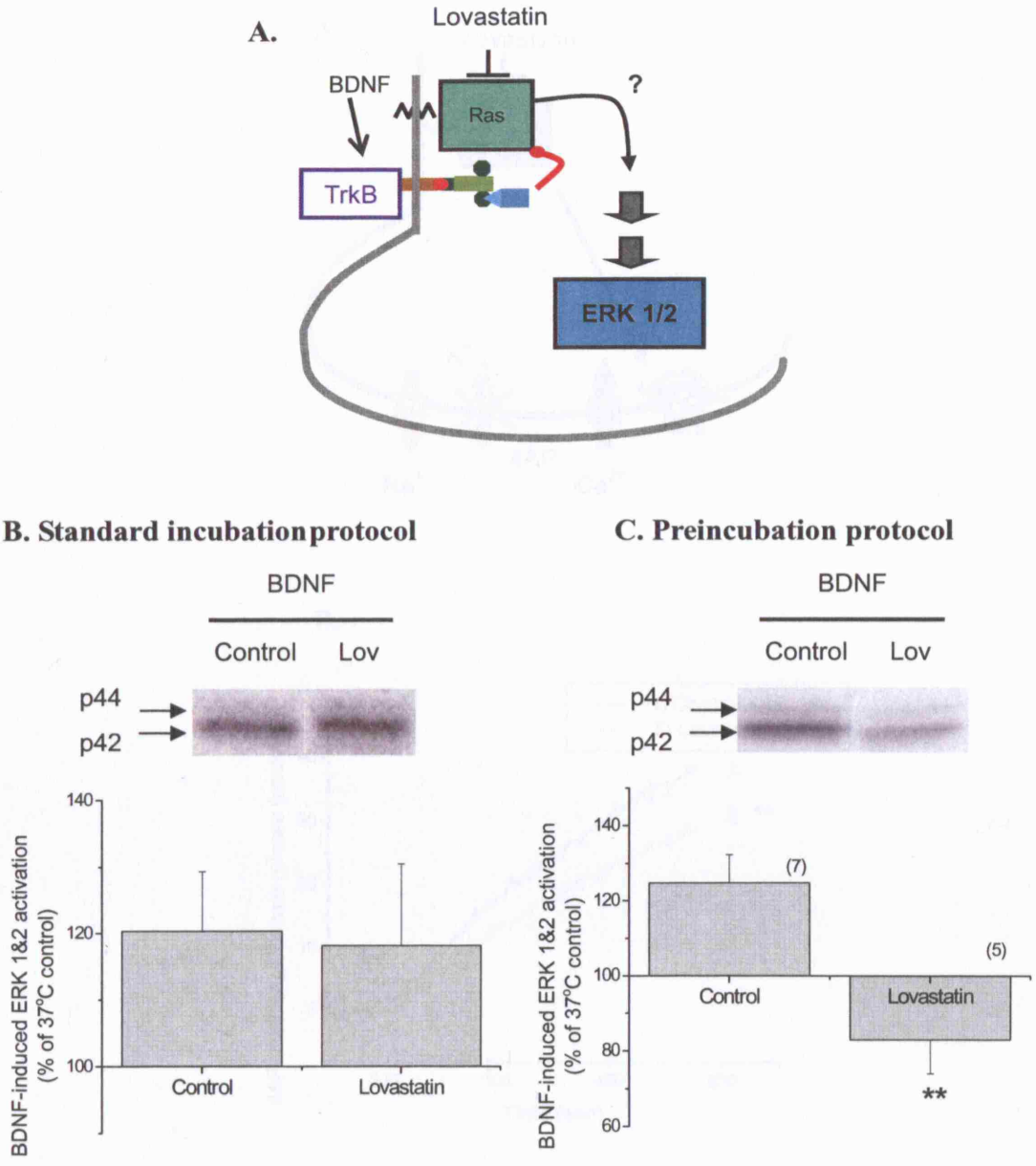



**Figure 3.5. Effect of lovastatin, a Ras inhibitor, on BDNF-mediated ERK 1&2 activation.**

**A.** Scheme showing proposed effect of lovastatin on BDNF-stimulated activation of ERK. **B.** Phosphoimages and quantification of phospho-ERK 1&2 levels after standard incubation with lovastatin. **C.** Phosphoimages and quantification of phospho-ERK 1&2 after preincubation with lovastatin. Synaptosomes (0.3 mg/ml) were incubated with 200ng/ml BDNF. Samples were treated with (Lov) or without (Control) 10μM lovastatin depending on the drug incubation protocol as indicated in the Materials and Methods section. **$p<0.01$ for long incubation of the drug (BDNF/Lov) compared to BDNF/37°C control (unpaired Student t-test). Experiments were carried out with eight synaptosomal preparations for standard drug incubation protocol (n=8) and seven or five for preincubation.

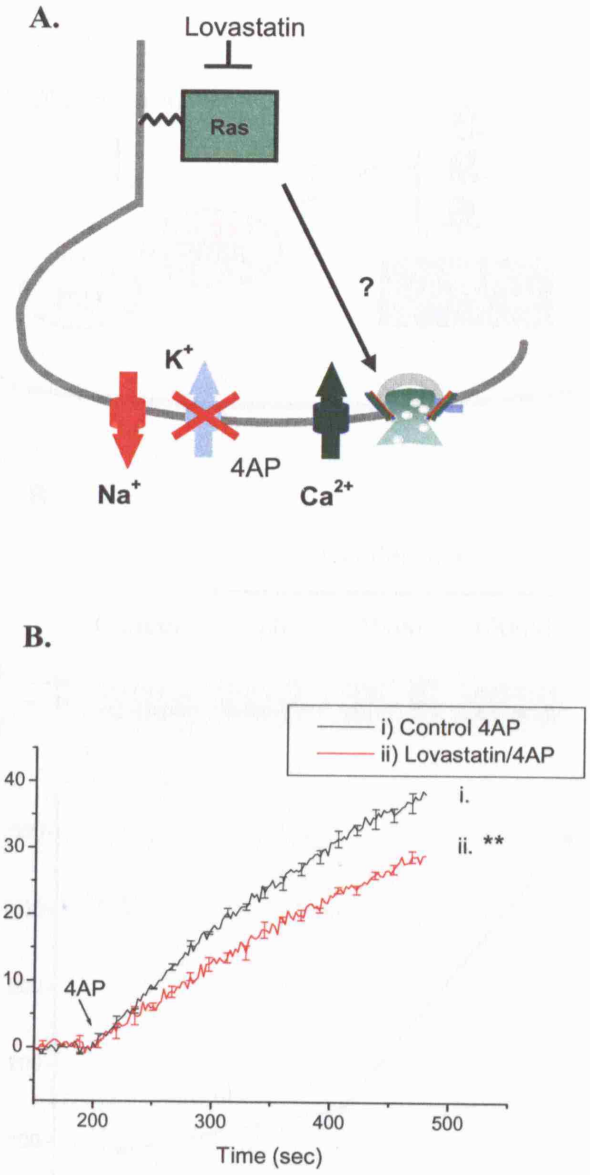



**Figure 3.6. Effect of lovastatin on 4AP-induced glutamate release.**

**A.** Scheme showing proposed effect of lovastatin on glutamate release to be tested. **B.** Glutamate release: i) Control 4AP; ii) Lovastatin/4AP. Synaptosomes (0.1 mg/ml) were preincubated in the absence (i) and presence (ii) of 10μM lovastatin for 20 minutes, pelleted and resuspended before following the standard incubation protocol using on-line fluorimetry as indicated in the Materials and Methods section. **$p<0.01$ compared to 4AP control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with three independent synaptosomal preparations (n=3).

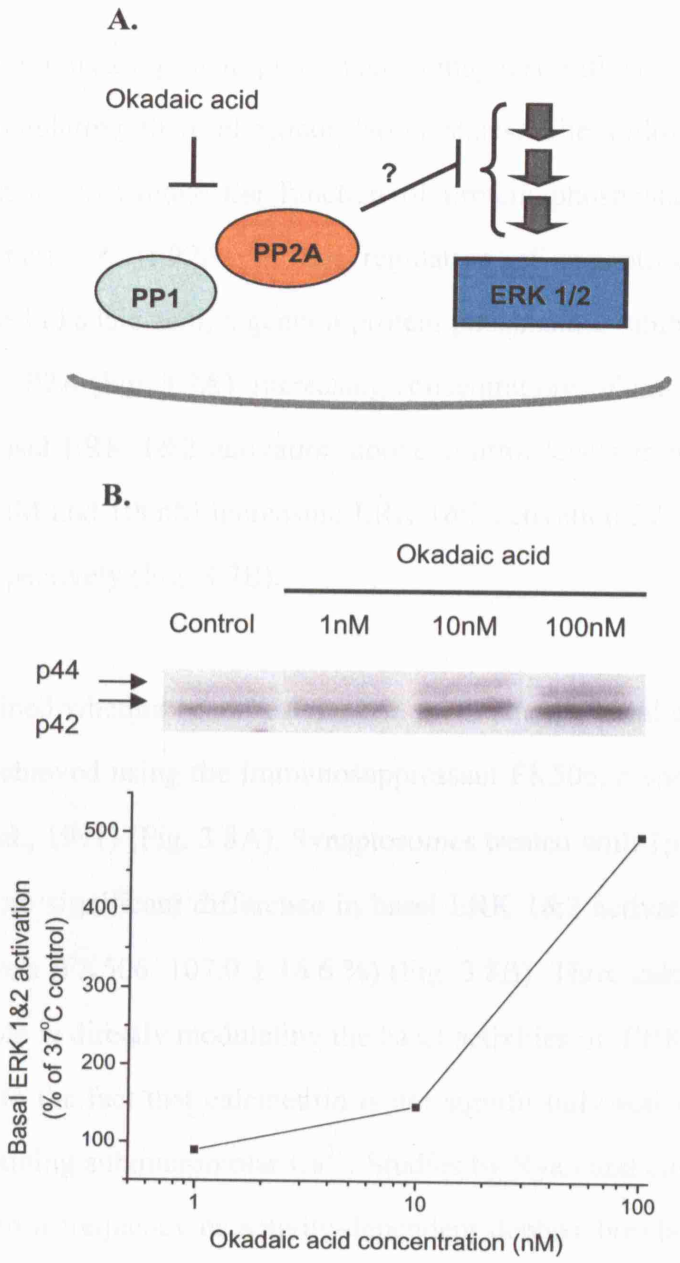



**Figure 3.7. Dose-dependency of okadaic acid, a general protein phosphatase inhibitor, on basal ERK 1&2 activation. A.** Scheme showing proposed effect of okadaic acid on basal activation of ERK 1&2. **B.** Phosphoimages and quantification of phospho-ERK 1&2 levels. Synaptosomes (0.3 mg/ml) were incubated in the absence (control) and presence of increasing concentrations of okadaic acid (1, 10, 100nM) at the start of the experiment and 1mM $CaCl_2$ was added at 3 min. The experiment was carried out with one synaptosomal preparation (n=1).

synaptosomes.

Apart from protein kinases, protein phosphatases may also influence the activation of ERK 1&2 by regulating their phosphorylation states. The following experiment, therefore, sought to investigate the function of protein phosphatases 1 (PP1) and protein phosphatase 2A (PP2A) in the regulation of synaptosomal ERK 1&2 activities. We used okadaic acid, a general protein phosphatase inhibitor that potently inhibits PP1 and PP2A (Fig. 3.7A). Increasing concentrations of okadaic acid effected an increase in basal ERK 1&2 activation above control levels in a dose-dependent manner, with 10nM and 100nM increasing ERK 1&2 activation 37.3 % and 397.2 % above control respectively (Fig. 3.7B).

Next, we determined whether or not calcineurin modulate the basal activities of ERK 1&2. This was achieved using the immunosuppressant FK506, a specific calcineurin inhibitor (Liu et al., 1991) (Fig. 3.8A). Synaptosomes treated with 1μM FK506 for 10 minutes showed no significant difference in basal ERK 1&2 activation compared to 100% control levels (FK506, 107.0 ± 16.6 %) (Fig. 3.8B). Thus, calcineurin does not seem to have a role in directly modulating the basal activities of ERK 1&2. However, this may be due to the fact that calcineurin is not significantly activated under basal conditions maintaining submicromolar $Ca^{2+}$. Studies by Ryan and coworkers, indeed, have demonstrated a frequency or activity-dependent dephosphorylation of synapsin site 4,5,6 by calcineurin (Chi et al., 2003). This is consistent with the known activation of this protein phosphatase by elevated $Ca^{2+}$ during neuronal stimulation (Yakel, 1997). Accordingly, to investigate whether modulation of ERK 1&2 by calcineurin occurs following its activation by $Ca^{2+}$, the $Ca^{2+}$ ionophore ionomycin was used to effect direct $Ca^{2+}$ entry into the synaptosomes (Fig. 3.9A). This obviates the involvement of calcineurin on upstream ionic loci (Czirjak et al., 2004; Ledoux et al., 2003; Norris et al., 2002) through bypassing VGCCs and channels involved in

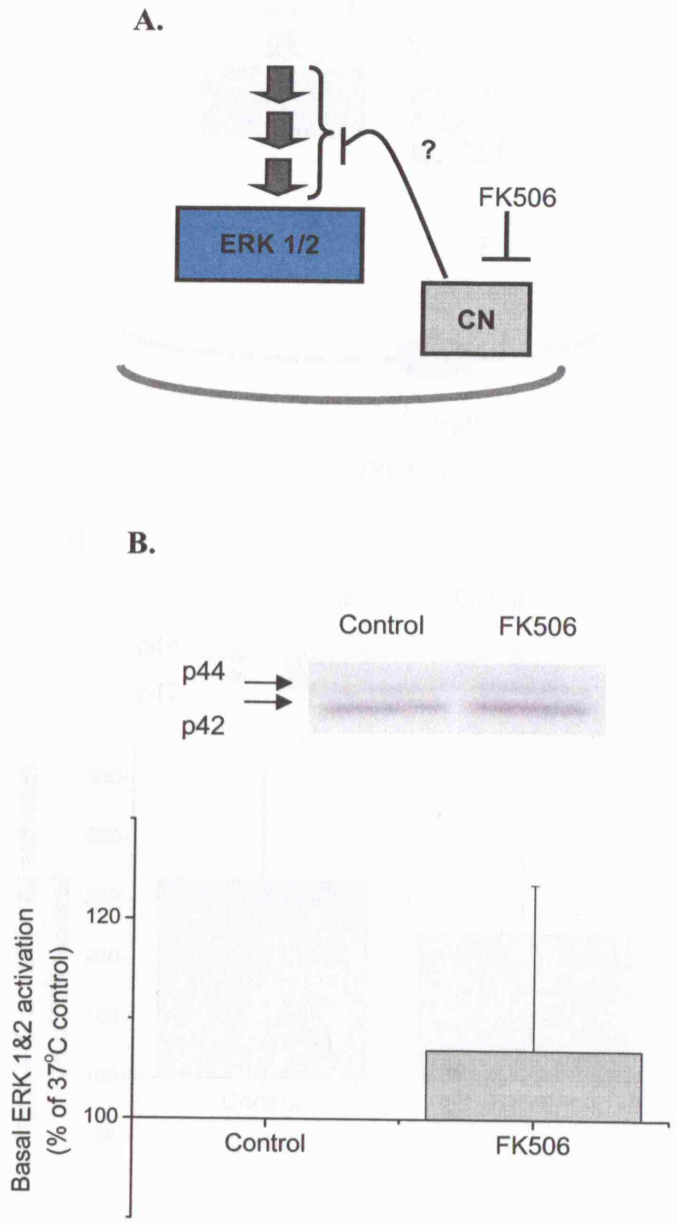



**Figure 3.8. Effect of FK506, a calcineurin (CN) inhibitor, on basal ERK 1&2 activation.**

**A.** Scheme showing proposed effect of FK506 on ERK 1&2 activation during basal conditions. **B.** Phosphorimages and quantification of basal phospho-ERK 1&2 levels with or without addition of FK506. Synaptosomes (0.3 mg/ml) were incubated in the absence (Control) and presence of 1μM FK506 (FK506) with 1mM $CaCl_2$ added at 3 min. Data represent means ± SEM of experiments carried out with six independent synaptosomal preparations (n=6)

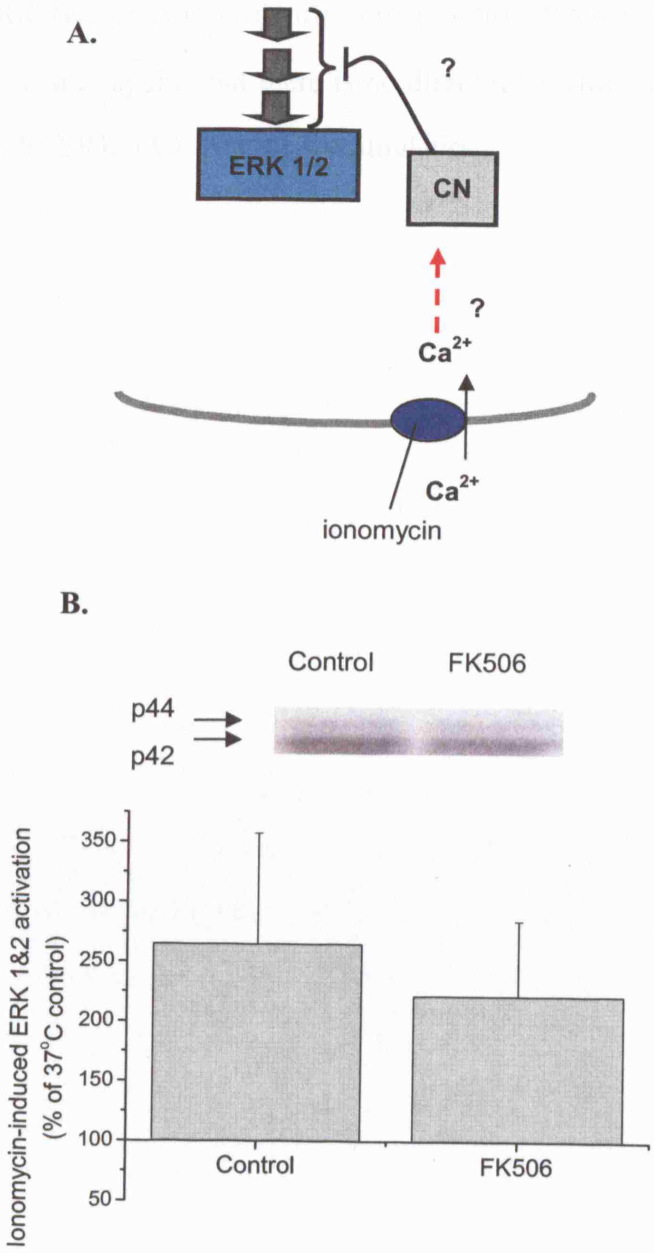



**Figure 3.9. Effect of FK506 on $Ca^{2+}$-dependent ERK 1&2 activation effected with ionomycin.**

**A.** Scheme showing proposed effect of FK506, a calcineurin (CN) inhibitor, on ionomycin-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of ionomycin-induced $Ca^{2+}$-dependent phospho-ERK 1&2 levels. Synaptosomes (0.3 mg/ml) were incubated in the absence (control) and presence of 1μM FK506, with 50μM EGTA added at 3 min and 5μM ionomycin added at 4 min. Calcium (500μM buffered-free $CaCl_2$) was added at 10 min to effect $Ca^{2+}$-dependent activation of ERK 1&2. Data represent means ± SEM of experiments carried out with seven independent synaptosomal preparations (n=7).

membrane excitability. Figure 3.9B reveals that the addition of FK506 did not affect $Ca^{2+}$-evoked ERK 1&2 activation significantly (control, 265.0 ± 93.0%; FK506, 222.0 ± 62.7 %), suggesting, again, that there is no direct interaction or cross-talk between calcineurin and the ERK 1&2 even upon stimulation.

### 3.4. Discussion

The results in this chapter show that the neurotrophin BDNF potentiated 4AP-induced release of glutamate from cerebrocortical nerve terminals, consistent with previous studies by Jovanovic *et al.* (Jovanovic et al., 2000), which implicated a facilitatory role of BDNF in neurotransmitter glutamate release. In addition, studies in other systems have also shown the effect of BDNF resulting in an enhancement of presynaptic release and synaptic transmission (Goggi et al., 2003; Goggi et al., 2002; Marmigere et al., 2001; Kang and Schuman, 2000; Li et al., 1998; Knipper et al., 1994). These studies have suggested the modulation of synaptic activity by BDNF can occur at many levels including the upregulation of the expression of synaptic proteins (Matsumoto et al., 2006), the recruitment of synaptic vesicles or docking proteins (Tyler et al., 2002; Tyler and Pozzo-Miller, 2001; Jovanovic et al., 2000; Pozzo-Miller et al., 1999), the activation of a PI3K or ERK pathway (Jovanovic et al., 2000; Gottschalk et al., 1999; Pozzo-Miller et al., 1999) or the regulation of sodium channels to affect excitability (Urbano and Buno, 2000). Other reports have suggested that BDNF works by two principal mechanisms, both of which involve the elevation of cytosolic $Ca^{2+}$: i) by modulation of VGCC to increase $Ca^{2+}$ influx (Baldelli et al., 2000; Baldelli et al., 1999); ii) by the activation of $Ca^{2+}$ stores to affect cytosolic levels of $Ca^{2+}$ (Li et al., 1998; Stoop and Poo, 1996), thereby increasing the probability of vesicular docking and neurotransmitter output (Goggi et al., 2003; Goggi et al., 2002; Marmigere et al., 2001; Kang and Schuman, 2000; Blanquet and Lamour, 1997). Given the $Ca^{2+}$ requirement of a low-affinity exocytotic trigger, it is unlikely that the release of intraterminal $Ca^{2+}$, by itself, would sufficiently raise the bulk cytosolic $Ca^{2+}$ to support exocytosis (Nicholls et al., 1987). In addition, BDNF has been shown to facilitate ionomycin-induced glutamate release through a mechanism independent of $Ca^{2+}$ entry and modulation of membrane excitability (Jovanovic et al., 2000). This implies that neurotrophin can also act at loci downstream of $Ca^{2+}$ entry, possibly at the level of the release machinery by directly

increasing the levels and activation of exocytosis-related proteins. In the present study using phosphorylation/ activation-state specific antibodies, our results revealed the presence of the two isoforms of ERK, ERK 1&2, in synaptosomes, which are sensitive to the MEK inhibitor, PD98059. Given that a parallel increase in the phosphorylation of ERK 1&2 was observed following BDNF stimulation, our results are in agreement with the cascade previously proposed where BDNF-induced modulation of neurotransmitter release is mediated via a TrkB/ERK/synapsin-dependent pathway (Jovanovic et al., 2000).

Considering the modulatory role of ERK 1&2 underlying neurotransmitter release, the particular pathways by which ERK can be activated are of primary importance. Data showed that during basal conditions, the levels of ERK 1&2 phosphorylation are high, with these basal activities of ERK 1&2 being subject to regulation by specific stimuli, including BDNF as discussed earlier. As many studies have implicated the role of small G-protein Ras in the "classic" ERK 1&2 signalling cascade (Wellbrock et al., 2004; Bos, 1998; Wood et al., 1992), we sought to address the extent to which Ras activation contributes to the BDNF-induced ERK 1&2 activation in the presynaptic terminal. Previously, attempts to establish levels of Ras activation in synaptosomes using antibodies raised against the GTP-binding domain (GBD) peptide of downstream Raf-1 kinase found a high level of Ras-GTP present in the basal condition (Davies and Sihra, personal communication). Although this is in agreement with the high level of basal ERK 1&2 activities observed here, a direct relationship between the activation of Ras and the ERK signalling cascade has not been shown. By utilizing lovastatin, a Ras inhibitor that specifically inhibits the activation of Ras from its GDP state, our results show that preincubation with the inhibitor leads to a complete abolition of BDNF-induced ERK 1&2 activation, suggesting an essential Ras-dependence of signalling in the nerve terminal BDNF-ERK pathway. This is further supported by a concomitant reduction of depolarisation-evoked glutamate release following the addition of lovastatin, indicating an important role of Ras in the

regulation of neurotransmitter release. These data are consistent with the model of the presynaptic BDNF-induced ERK signalling cascade involving TrkB activation, recruitment of adaptor protein (Grb2) and guanine nucleotide exchange factor (Sos), and the activation of Ras from its inactive GDP state to its active GTP state. The activated Ras, in turn, promotes activation of its effector, Raf-1 and/or B-Raf (which has been found at high levels in synaptosomes (Davies, 2004)) to transduce the signal to downstream MEK and ERK activation (York et al., 1998). Thus, the data presented here suggest Ras likely represents a major link between TrkB and ERK 1&2 activation in the nerve terminal. However, in order to better understand the involvement of the small GTP-binding proteins, further work will be required to precisely identify the downstream effectors of Ras, i.e. whether it is Raf-1 or B-Raf that is the major MEK kinase in the BDNF-Ras-ERK pathway. If it is the latter case, experiments to consider the involvement of Rap1 in synaptosomes will also help to address this issue.

Having established the upstream Ras-dependent regulation of ERK 1&2, it is important to consider the factors that may control the lifetime of ERK activities through modulation of the phosphorylation state of ERK 1&2. Apart from protein kinases, another possibility by which activities of ERK 1&2 can be regulated is by the actions of protein phosphatases. Results presented here suggest PP1 and PP2A are the protein phosphatases that exert control on ERK 1&2 during basal conditions. This is evinced by the addition of varying concentrations of okadaic acid, a general PP1 and PP2A inhibitor, which showed potentiation of basal ERK 1&2 activation in a dose-dependent manner. Indeed, studies have demonstrated that PP2A can directly dephosphorylate the threonine residues of ERK 1&2 (Alessi et al., 1995). The negative modulation of ERK 1&2 by PP2A is further supported by experiments in other systems showing that hyperactivation of ERK can be mediated by the silencing of PP2A Bα and Bδ subunits (Van Kanegan et al., 2005). In addition, findings that inhibition of PP2A could cause the activation of MEK 1&2 and that MEK 1&2

inhibitor could abolish ERK phosphorylation induced by the PP2A inhibitor suggest that MEK may serve as a control for ERK signalling in response to PP2A action (Mao et al., 2005). Thus, PP2A may regulate ERK 1&2 signalling by downregulating regulators upstream of MEK 1&2 as well as acting on ERK 1&2 directly. This inhibitory role of PP2A in the presynaptic nerve terminal is also in agreement with the ability of PP2A to dephosphorylate synapsin (Jovanovic et al., 2001) and neurofilaments (Strack et al., 1997b), in so doing, provide a mechanism for negatively regulating presynaptic release by modulating the ability of synapsin to associate with SVs and the cytoskeletal elements. Indeed, studies have reported the presence of PP2A in the nerve cytosol (Sontag, 2001) and its involvement in regulating neurotransmitter release (Sistiaga and Sanchez-Prieto, 2000). Thus, although a number of studies have suggested the facilitatory role of PP2A on Ras signalling via dephosphorylating hyperphosphorylated or desensitised Raf-1 (Adams et al., 2005; Dougherty et al., 2005) or by dephosphorylating KSR (Ory et al., 2003), data here implicate PP2A as a negative regulator of ERK 1&2.

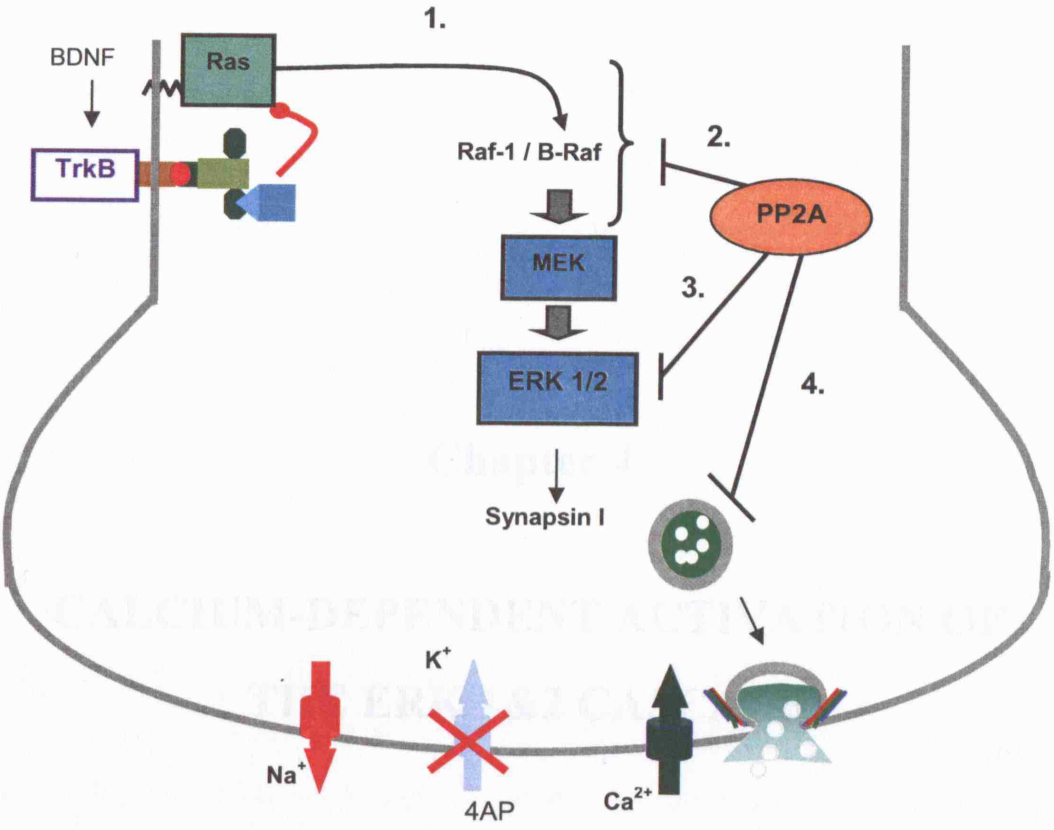
With respect to PP1, while the foregoing data based on the action of okadaic acid could also suggest the regulation of ERK 1&2 activities by PP1, there is a line of evidence suggesting that PP1 is localised primarily in post-synaptic sites rather than presynaptically (Allen et al., 1997). Furthermore, there has been a report showing that the key phosphorylation sites of synapsin I are substrates of PP2A, but not PP1 (Jovanovic et al., 2001). Notwithstanding these data, we cannot completely rule out the potential regulation of basal ERK 1&2 activation by PP1 given that, more recently, the γ1 isoform of PP1 has been shown to be located to presynaptic terminals in the cortical pyramidal cells (Strack et al., 1999). In addition, the modulation of PP1 is known to be complex. In that respect, modulation of ERK 1&2 activation following activation of PP1 may depend on the activity of PP2B or calcineurin. Thus, although PP1 is not directly affected by intracellular $Ca^{2+}$, modulation by inhibitor-1, which is activated by PKA-dependent phosphorylation, can be abrogated by the $Ca^{2+}$-

dependent activation of calcineurin (Yakel, 1997; Torii et al., 1995; Mulkey et al., 1994). However, given that our results also showed that calcineurin does not have a role in modulating basal/ionomycin-induced ERK 1&2 activities, with the demonstration that the addition of the calcineurin inhibitor FK506 resulted in no significant effect on either basal and stimulated ERK 1&2 activation, it is likely that neither PP1 nor calcineurin have a role on ERK 1&2 signalling as regulators. Thus, the facilitation of ERK 1&2 seen with the addition of okadaic acid appears to directly relate to the negative modulation of ERK 1&2 activation by PP2A rather than PP1. However, the use of another general PP1 and PP2A inhibitor which displayed a higher affinity to PP2A than to PP1, such as calyculin A (Takai et al., 1995), the newer PP2A inhibitors such as fostriecin and/or the PP1 inhibitory peptide inhibitor-1 or 2 (Oliver and Shenolikar, 1998), may be useful in more directly confirming or refuting the function of PP1.

On balance, the results here indicate that PP2A, but not PP1 or calcineurin, acts predominantly as a gatekeeper of basal ERK activity, enabling the longevity of ERK signalling to be finely controlled to generate a precise biological response. These findings appear to fit in well with the current model of synapsin modulation by ERK 1&2 and the phosphatases that are involved in opposing changes of phosphorylation and dephosphorylation of synapsin during stimulation. During basal conditions, synapsins are dephosphorylated by PP2A on synapsin phosphosites 1,2,3 such that dephosphosynapsins can associate with membranes of SVs and neurofilaments. In addition, our data here also suggest that PP2A may result in the dephosphorylation of presynaptic ERK 1&2, thereby downregulating their activities. This contributes to the overall phosphorylation state of synapsin by reducing the ability of ERK mediating phosphorylation on synapsin at its phosphosite 4,5,6. Upon $Ca^{2+}$ influx, the synapsins are phosphorylated by the increasing activities of protein kinases such as PKA and CaMKI and II at sites 1,2,3 and dephosphorylated by calcineurins at ERK 1&2-dependent sites 4,5,6 (Jovanovic et al., 2001). This enables the synapsins to dissociate

from the SVs and cytoskeletal matrix and, in so doing, increase the number of SVs available for release. Although our data here indicate that neither PP1 nor calcineurin are likely to function to directly affect ERK 1&2 activation, the presence of $Ca^{2+}$-dependent activation of calcineurin in the nerve terminal, nevertheless, provides an inhibitory constraint on presynaptic release by acutely limiting the ERK cascade impinging on synapsin through dephosphorylating synapsin on phosphosites 4,5,6 during high frequency stimulation (Chi et al., 2003). Confirmation of this hypothesis will require future studies looking directly at the phosphorylation of synapsins at the ERK 1&2-dependent phosphosites 4,5,6 under the conditions established herein.

In conclusion, the ERK 1&2 signalling in the nerve terminal has been demonstrated to be specifically stimulated by neurotrophins through TrkB activation in a *Ras-dependent* manner. Moreover, the basal ERK 1&2 activity appears to be temporally controlled by PP2A. The schematic diagram below **(Schematic 3.4)** summarises the possible mechanisms of neurotrophin and protein phosphatase mediated regulation on ERK 1&2 activation in the nerve terminal.




**Schematic 3.4. Neurotrophin and protein phosphatase-mediated regulation of ERK 1&2 signalling pathways underlying the control of neurotransmitter release.** 1.) BDNF-stimulation of TrkB receptor results in the recruitment of adaptor Grb2 and Sos to cause the activation of GTP-binding protein Ras, and thereby, leading to the subsequent MEK 1&2 and ERK 1&2 activation via a Raf-1 or B-Raf pathway; 2.) PP2A may effect negative regulation of ERK 1&2 by dephosphorylating regulators upstream of MEK 1&2; 3.) PP2A may directly dephosphorylate ERK 1&2 on phosphothreonine residues to lead to downregulation of ERK 1&2 activities; 4.) PP2A may dephosphorylate synapsin I and cytoskeletal elements to enable SVs to enter into the RRP for exocytosis.

# Chapter 4

# CALCIUM-DEPENDENT ACTIVATION OF THE ERK 1&2 CASCADE

## 4.1. Introduction

Neurotransmitter release has long been established to be dependent on $Ca^{2+}$ influx as a result of depolarisation (Sihra et al., 1993; Smith and Augustine, 1988; Augustine et al., 1987). It is commonly believed that following depolarisation, $Ca^{2+}$ entry into the nerve terminal through VGCCs is sensed by the vesicular protein synaptotagmin, which in turn, triggers release by allowing interaction of the SNARE proteins so that the SVs fuse with the plasma membrane to release their contents. During stimulation, sub-synaptic $Ca^{2+}$ concentrations can be raised to a level as high as 100μM where changes are localised to the active zone close to the site of $Ca^{2+}$ entry (Augustine, 2001; Hsu et al., 1996; Heidelberger et al., 1994). Ultrastructural studies showed that multiple VGCC subtypes exist in the presynaptic terminal. A number of studies have demonstrated that release is mainly associated with N- and P/Q- types VGCCs (Reid et al., 2003; Spafford and Zamponi, 2003; Timmermann et al., 2002; Wu et al., 1999; Reid et al., 1998; Wheeler et al., 1994). In addition, the R-type VGCCs, locating at perisynaptic sites (Wu et al., 1999), have been shown to contribute partially to exocytosis (Kamp et al., 2005; Gasparini et al., 2001).

Apart from having a role in the fusion stage of exocytosis, $Ca^{2+}$ is also an important second messenger in neuronal signalling mechanisms. Studies have shown that increases in intracellular $Ca^{2+}$ can regulate a number of ion channels in the plasma membrane (Catterall, 2000) as well as signalling molecules including calpain (Friedrich, 2004), protein kinase C (PKC) (Hori et al., 1999; Parfitt and Madison, 1993) and $Ca^{2+}$/CaM kinase II (CaMKII) (Griffith, 2004; Colbran and Brown, 2004; Lisman et al., 2002). Indeed, the importance of $Ca^{2+}$ has been highlighted in studies where $Ca^{2+}$ restriction and/or dysregulation has been shown to be directly linked to ischaemic injury (Kondo et al., 2005; Won et al., 2002), excitotoxity (Salinska et al., 2005) and Alzheimer's disease (Smith et al., 2005; Monaco, III, 2004).

Presynaptically, $Ca^{2+}$ has been implicated in the modulation of synapsin phosphorylation by a number protein kinases and phosphatases that have been shown to be dependent on $Ca^{2+}$. Among these, activation of CaMKI and CaMKII, which mediate phosphorylation of synapsin I at phosphosites 1 and 2/3 respectively, has been shown to require $Ca^{2+}$ and calmodulin (CaM) (Colbran and Brown, 2004; Lisman et al., 2002). In addition, the activity of calcineurin (Yakel, 1997; Klee et al., 1998), as a Ser/Thr phosphatase involved in dephosphorylating synapsin at phosphosites 4,5,6 upon nerve stimulation, has also been shown to be $Ca^{2+}$/CaM-dependent (Chi et al., 2003; Jovanovic et al., 2001). Thus, the ability of synapsin to promote SV availability for release greatly depends on the opposing changes in its phosphorylation state in response to intraterminal $Ca^{2+}$ dynamics.

Studies have revealed that phosphorylation of synapsin I at phosphosites 4,5,6 is under tonic modulation by ERK 1&2 in the mechanism underlying the control of neurotransmitter release (Chi et al., 2003; Jovanovic et al., 2001; Jovanovic et al., 2000). This is evident from glutamate release studies in the cerebrocortical nerve terminal showing that neurotrophin BDNF can facilitate depolarisation-induced glutamate release via a TrkB/ERK/synapsin-dependent pathway (Jovanovic et al., 2000). Although direct $Ca^{2+}$-dependent signalling to ERK 1&2 in the presynaptic nerve terminal has not been shown, ERK 1&2 activation independent of neurotrophic-mediated mechanisms has been demonstrated postsynaptically and in a number of non-neuronal systems, in response to increases in cytoplasmic free $Ca^{2+}$ (Corvol et al., 2005; Egea et al., 2000; Egea et al., 1999). There is evidence that depolarisation-dependent increases in $Ca^{2+}$ levels can activate Ras to lead to stimulation of the ERK signalling cascade (Baldassa et al., 2003; Agell et al., 2002; Finkbeiner and Greenberg, 1996; Rosen et al., 1994). $Ca^{2+}$ may activate Ras via at least two mechanisms: i) by binding to the Pyk2/Src complex (Dikic et al., 1996; Rosen and Greenberg, 1996; Lev et al., 1995); ii) by activating Ras regulators such as RasGEF which are known to be $Ca^{2+}$-dependent (Aspenstrom, 2004; Walker et al., 2003;

Farnsworth et al., 1995; Ebinu et al., 1998). Furthermore, an important role of $Ca^{2+}$ in ERK 1&2 signalling has also been shown in neurotrophin-mediated activation of ERK 1&2 where permissive increases of $Ca^{2+}$ are required in the system for BDNF-mediated activation of the ERK 1&2 cascade to occur (Goggi et al., 2002; Kang and Schuman, 2000).

This chapter aims to examine the effect of depolarisation and $Ca^{2+}$on ERK 1&2 activation in the presynaptic nerve terminal. Contingent to showing a $Ca^{2+}$-dependency of ERK 1&2 activation, we sought to investigate whether the $Ca^{2+}$-dependency of ERK 1&2 activation seen was due to a direct effect of $Ca^{2+}$ on the ERK 1&2 cascade or via an indirect action involving membrane excitability and modulation at the level of $Ca^{2+}$ entry. In addition, the source of $Ca^{2+}$ was determined to ascertain whether ERK 1&2 activation was associated with external $Ca^{2+}$ entry or intracellular $Ca^{2+}$ released from stores present in the nerve terminal. Finally, the extent to which the $Ca^{2+}$ dependent activation was attributable to neurotrophin-mediated ERK 1&2 signalling was explored.

## 4.2. Materials and Methods

### 4.2.1. Synaptosomal preparation

Synaptosomes were prepared as described in section 2.1.1. Mouse synaptosomes were prepared from R-type VGCC knockout animals as first generated by Pereverzev *et al* (1998) and genotyped by tail-slip and Southern blot analysis. Synaptosomes from age and weight matched knockout and wildtype animals were compared.

### 4.2.2. Standard incubation for ERK 1&2 activation/phosphorylation

Standard protocol was followed as indicated in section 2.4.1 where synaptosomal pellets were resuspended in HBM containing 1mg/ml BSA and incubated for a total of 10 minutes. BAPTA-AM (100μM), thapsigargin (1μM) or SNX-482 (100nM) was added at the start of the incubation. $CaCl_2$ (1mM) or EGTA (100μM) was added at 3 min before depolarisation with secretagogues 4AP (100μM or 1mM) or KCl (5mM, 10mM or 30mM) at 10 min. Reactions were terminated with the addition of sample buffer 1 min after the secretagogue, unless otherwise stated.

### 4.2.3. Protocol measuring $Ca^{2+}$-dependency of ERK1&2 with ionomycin and $Ca^{2+}$-buffers

Synaptosomes were incubated in the HBM buffer containing 1mg/ml BSA for 10 minutes as described in section 2.4.3. EGTA (50μM) as $Ca^{2+}$-buffer was added at 3 min followed by the addition of 5μM ionomycin at 4 min. Varying concentrations of free calcium (0, 1, 10, 50, 100 or 500μM) were then added at 10 min before the reactions were stopped with the addition of sample buffer. Drugs of interest were added at the times indicated in the figure legends.

### 4.2.4. BAPTA-AM/EGTA assay protocol

Synaptosomes were preincubated at 37°C in HBM buffer containing 1mg/ml BSA and

1mM $CaCl_2$ as indicated in section 2.4.4. DMSO (1%, as control) or BAPTA-AM (100μM) was added at 10 min and synaptosomes were preincubated for a further 20 min before the suspension was centrifuged in a microfuge for 30 sec at 10,000xg and supernatants removed. Pellets were cooled at 4°C for 5 min. Synaptosomal pellets were then resuspended and incubated normally as in the standard incubation protocol with the addition of 1mM $CaCl_2$ or 100μM EGTA at 3 min and secretagogue 4AP at 10 min. Samples were stopped with sample buffer and subjected to SDS-PAGE, protein electrotransfer and immunoblotting.

### 4.2.5. SDS-PAGE and Immunoblotting

Samples were separated on SDS-PAGE and subjected to immunoblotting as described in detail in sections 2.4.5 and 2.4.6.

### 4.2.6. Statistical analysis

Student's paired t-test was used to assess the confidence levels of significant differences between two sets of data. Confidence levels of differences between multiple sets of data were assessed using analysis of variance (ANOVA), followed by post hoc analysis with Dunnetts test.

### 4.2.7. Reagents

A stock of 5mM ionomycin was made in DMSO and working solution at a concentration of 500μM was obtained by dilution using HBM. Ionomycin was used at a 5μM final concentration.

A stock solution of 100mM BAPTA-AM was made in DMSO which was further diluted in HBM to give a working solution of 10mM. BAPTA-AM was used at a final concentration of 100μM.

Stock solution of EGTA (10mM) was obtained with water before further diluting to appropriate working solution. EGTA was used at final concentration of 50μM or 100μM.

A 1mM stock solution of thapsigargin was made with DMSO before dilution to a concentration of 100μM with HBM as a working solution. Thapsigargin was used at 1μM as final concentration.

A working solution of SNX-482 (1μM) was obtained using HBM. SNX-482 was used at a final concentration of 100nM.

## 4.3. Results

We determined whether ERK 1&2 signalling can be activated by depolarisation, independently of neurotrophin-mediated mechanisms in the nerve terminal (Fig. 4.1A). Synaptosomes were depolarised with secretagogues 4-aminopyridine (4AP) or high external potassium (KCl) (Nicholls, 2003; McMahon and Nicholls, 1991a), where 4AP acts as a $K^+$ channel blocker, whilst KCl induces direct depolarisation of the membrane by clamping the $K^+$-equilibrium potential at a depolarised level. Levels of ERK 1&2 activation were subsequently detected using phospho-state specific ERK 1&2 antibodies (Fig. 4.1A and 4.1B immunoblot inset). The effect of synaptic activity on the activation of ERK 1&2 was first determined by carrying out a time-course of depolarisation (1, 3, 5, 10 or 20 minutes) on synaptosomes. Figures 4.1A and 4.1B show the stimulation profiles of ERK 1&2 activation by 4AP and KCl. Whereas 4AP-induced activation of ERK 1&2 peaks at 5 min and began to diminish at 10 min (% above 37°C control: 1min, 29.8%; 3min, 39.7%; 5min, 64.0%; 10min, 37.6%; 20min, 21.6%) (Fig. 4.1A), KCl depolarisation predominantly evoked optimal activation at 3 min and by 5 min all KCl-induced activation of ERK 1&2 was abolished (% above 37°C control: 1min, 11.9%; 3min, 24.8%; 5min, 0.4%) and was further reduced below control at 20 min (27.1% below 37°C control) (Fig. 4.1B). As can be seen, both stimulation protocols of 4AP and KCl showed a time-dependent effect of depolarisation on ERK 1&2 activation. This suggests that the time and level of synaptic activity caused by the depolarising stimulus may be important in controlling the ERK 1&2 activities.

In order to study the upstream regulators of this depolarisation-induced ERK 1&2 activation, optimal conditions under which ERK 1&2 are activated needed to be established. Thus, depolarisation-induced ERK 1&2 activation to an intermediate level would allow both facilitatory and inhibitory effects to be examined. Results in Fig. 4.1A show that depolarisation of synaptosomes with 1mM 4AP for 1 min is

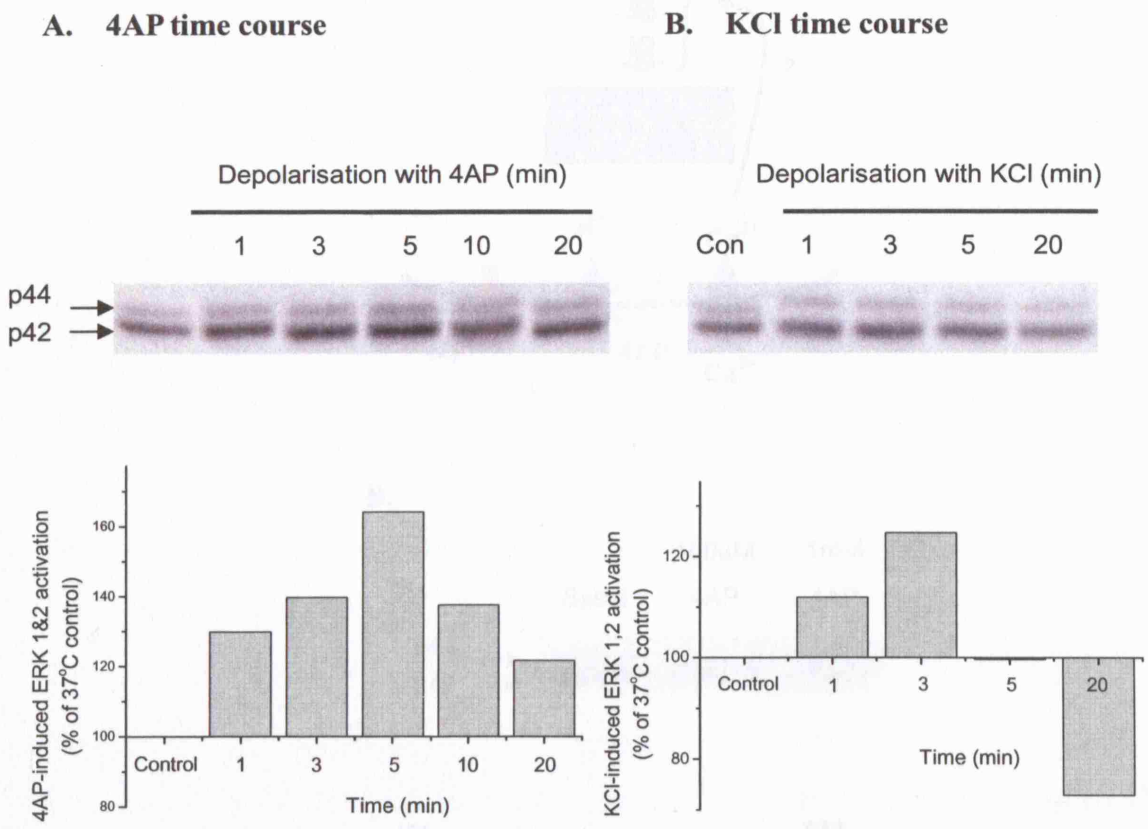



**Figure 4.1. Time course of depolarisation-induced ERK 1&2 activation.**

**A.** Phosphoimages and quantification of 4AP depolarisation time course on ERK 1&2 activation. **B.** Phosphoimages and quantification of KCl depolarisation time course on ERK 1&2 activation. Synaptosomes (0.3 mg/ml) were incubated at 37°C with 1mM of $CaCl_2$ added at 3 min followed by the addition of secretagogue (1mM 4AP or 30mM KCl) at 10 min. Synaptosomes were depolarised by 4AP or KCl for 1, 3, 5, 10 or 20 min before reaction was terminated. Samples were then separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes before being blotted with phospho-p44/42 ERK (Thr202/Tyr204) antibody (NEB) at 1:1000 dilution overnight at 4°C and probed using $^{125}$I-labeled Protein A, as indicated in the Materials and Methods section. Immuno-bands were detected and analysed with Typhoon phosphoimager (Molecular Dynamics). The experiment was carried out with one synaptosomal preparation (n=1).

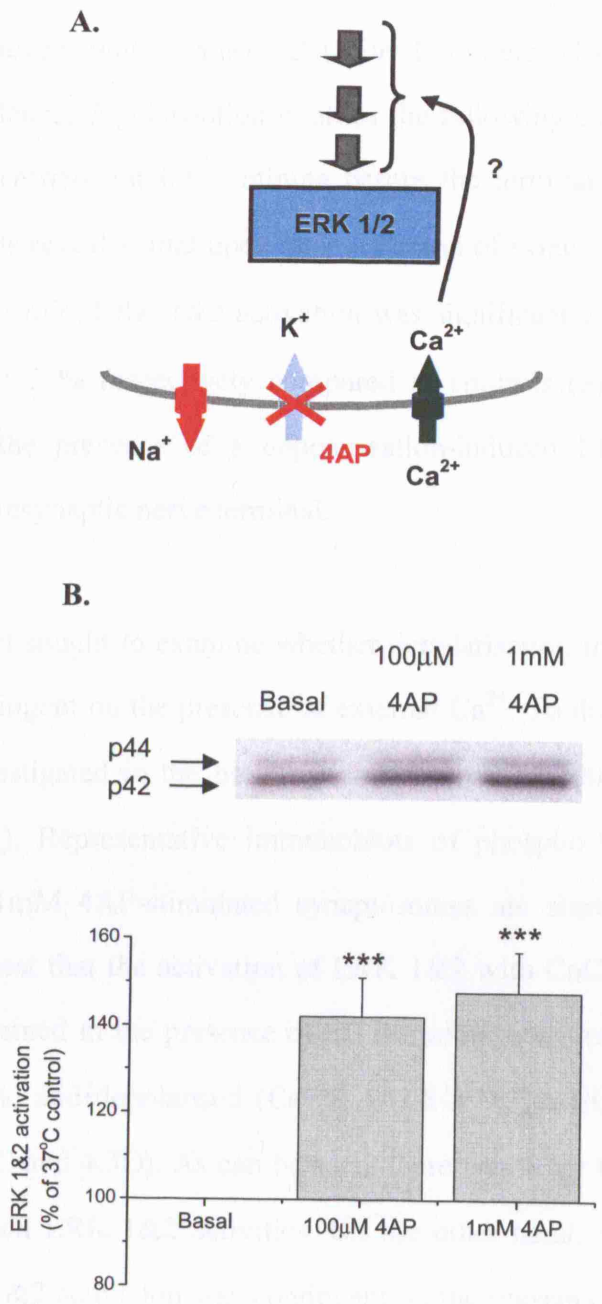



**Figure 4.2. ERK 1&2 activation stimulated by depolarisation with 4-aminopyridine (4AP).**

**A.** Scheme showing proposed effect of 4AP on ERK 1&2 activation. **B.** Phosphoimages and quantification of phospho-ERK 1&2 levels stimulated by 4AP. Synaptosomes (0.3 mg/ml) were incubated in the absence (Control) and presence of 4AP (100µM or 1mM 4AP), added at 10 min. ***$p<0.001$ compared to 100% 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with fifteen (n=15) and eight (n=8) independent synaptosomal preparations for 100µM 4AP and 1mM 4AP respectively.

capable of producing a sub-maximal stimulation of ERK 1&2 activation. Similarly, depolarisation achieved with 30mM KCl for 1 minute also reflects a robust stimulation level. Hence, depolarisation in all of the following experiments described in this thesis was carried out for 1 minute before the termination of the reaction. Immunoblot analysis revealed that upon depolarisation of synaptosomes with 100μM and 1mM 4AP for 1 min, ERK 1&2 activation was significantly increased by 42.2 ± 8.9 % and 47.8 ± 9.3 % respectively compared to controls (Fig. 4.2A and 4.2B), clearly indicating the presence of a depolarisation-induced ERK 1&2 signalling mechanism in the presynaptic nerve terminal.

The next experiment sought to examine whether depolarisation-induced activation of ERK 1&2 was contingent on the presence of external $Ca^{2+}$. To do this, stimulation of ERK 1&2 was investigated in the presence and absence of 100μM EGTA, a $Ca^{2+}$ chelator (Fig. 4.3A). Representative immunoblots of phospho-ERK 1&2 in basal conditions and in 1mM 4AP-stimulated synaptosomes are shown in Figure 4.3B. Averaged data suggest that the activation of ERK 1&2 with $CaCl_2$ was significantly higher than that obtained in the presence of EGTA under both resting ($CaCl_2$, 100%; EGTA, 84.3 ± 4.2%) and depolarised ($CaCl_2$, 147.8 ± 9.3%; EGTA, 97.1 ± 5.0%) conditions (Fig. 4.3C and 4.3D). As can be seen, there was a net $Ca^{2+}$-dependence of 15.7 ± 4.2% for basal ERK 1&2 activities. On the other hand, 50.8 ± 9.6% of the 4AP-induced ERK 1&2 activation was contingent on the presence of $Ca^{2+}$. This level of $Ca^{2+}$-dependence accounts for all of the 4AP/depolarisation-dependent increase of ERK 1&2 activation (Fig. 4.3E). This suggests a requirement for $Ca^{2+}$ in the 4AP/depolarisation-evoked activation of ERK 1&2 signalling.

Similar results were obtained with KCl-stimulated synaptosomes (Fig. 4.4A). Immunoblots are shown in Fig. 4.4B. Analysis showed that ERK 1&2 activation was dramatically increased when synaptosomes were depolarised in the presence of $CaCl_2$

(5mM, 208.0 ± 54.0%; 10mM, 186.0 ± 56.7%; 30mM, 147.0 ± 26.8%) (Fig. 4.4C) with the addition of EGTA reducing this activation level (Fig. 4.4D). However, for reasons that were not clear, there was substantial variability of the data with KCl, resulting in changes being statistically non-significant (Fig. 4.4E). It is notable that when EGTA was applied, the levels of KCl-induced ERK 1&2 activation were higher than those produced in the presence of $Ca^{2+}$ (only occurs with higher KCl concentrations; 10mM and 30mM). This appears to be consistent with previous studies which have shown that hyperphosphorylation of $^{32}$P-orthophosphate prelabelled presynaptic proteins occurred in the absence of $Ca^{2+}$ during KCl depolarisation (Sihra, personal communication). One reason for the unusual variability with respect to these experiments may be that KCl, by causing simultaneous firing of the ion channels, may cause a prolonged and high elevation of $Ca^{2+}$, such that reactions like the $Ca^{2+}$-dependent proteolysis by calpain may be activated (Nicholls et al., 1987). Considering these possibilities, KCl was not utilized further as an activator in the subsequent experiments.

Taken together, the foregoing experiments indicate that both basal and depolarisation-induced ERK 1&2 activation occur in a $Ca^{2+}$-dependent manner. Next, $Ca^{2+}$-dependent ERK 1&2 activation was examined directly by titrating intrasynaptosomal levels of $Ca^{2+}$ with increasing concentrations of added free $Ca^{2+}$, in the presence of the $Ca^{2+}$ ionophore, ionomycin (Blau and Weissmann, 1988) (Fig. 4.5A). Figure 4.5B shows that direct $Ca^{2+}$ entry effected by 5μM ionomycin led to an elevation in ERK 1&2 activation from its basal levels in a $Ca^{2+}$ dose-dependent manner. Statistical analysis (one-way ANOVA, followed by Dunnetts post-hoc test) shows that $Ca^{2+}$ concentration of 500μM was highly significant and different from the control and from other [$Ca^{2+}$]. As a control, the effect of ionomycin alone on basal ERK 1&2 activation was also evaluated and data demonstrate that ionomycin has no significant effect on the levels of basal ERK 1&2 activities, although some individual experiments did evince a tendency for ionomycin to increase basal ERK 1&2

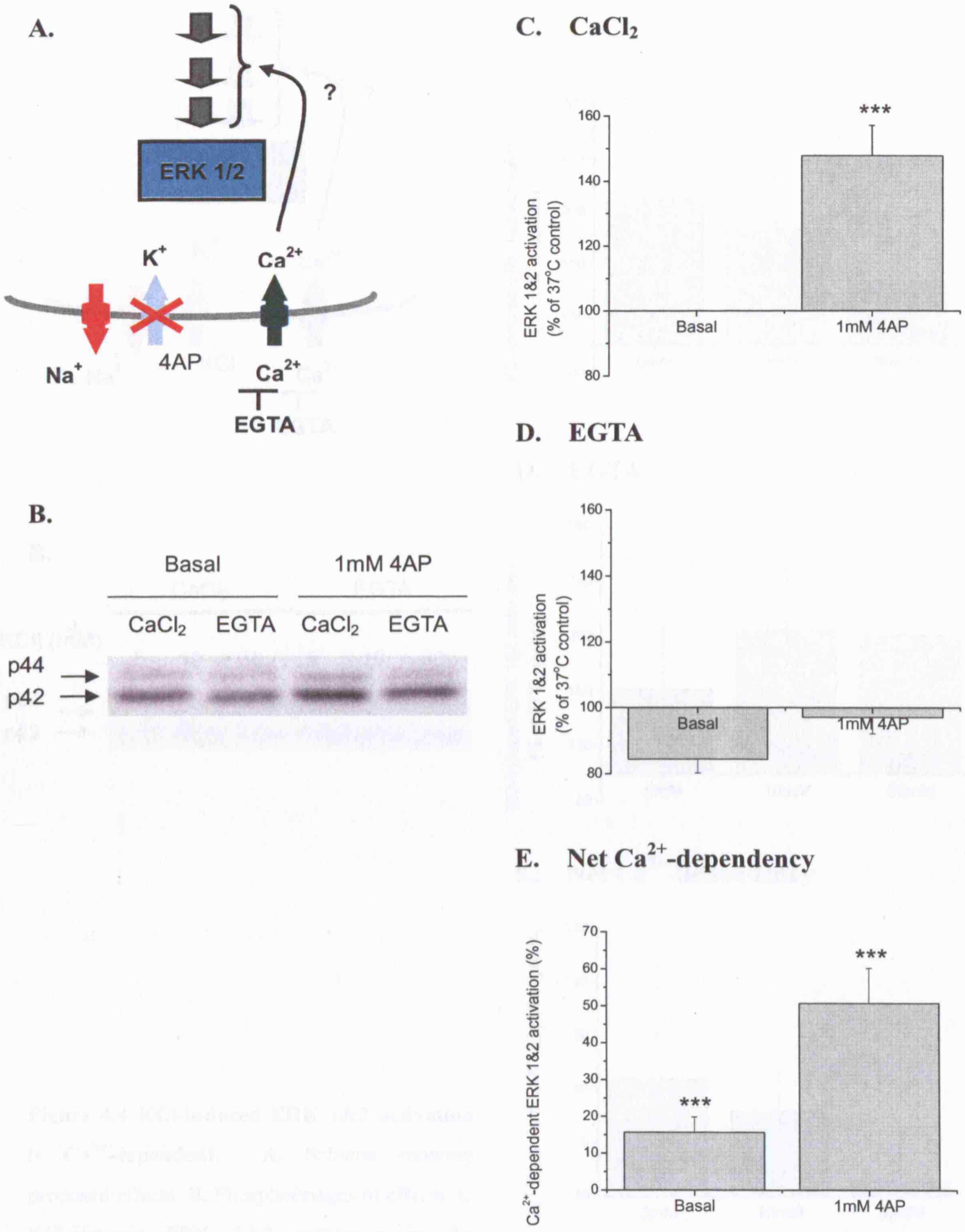



**Figure 4.3. Basal ERK 1&2 and 4AP-mediated ERK 1&2 activation are $Ca^{2+}$-dependent.**

**A.** Scheme showing proposed effects. **B.** Phosphoimages of effects. **C.** Basal and 4AP-induced activation of ERK 1&2 in the presence of $CaCl_2$. **D.** Basal and 4AP-induced ERK 1&2 activation in the absence of $CaCl_2$ (presence of EGTA). **E.** Net $Ca^{2+}$-dependency of ERK 1&2 activation. Synaptosomes (0.3 mg/ml) were incubated with 1mM of $CaCl_2$ or 100μM EGTA added at 3 min, followed by the depolarisation with 4AP (1mM) at 10 min. ***$p<0.001$ compared to 37°C control (unpaired Student t-test). Experiments carried out with eight synaptosomal preparations (n=8).

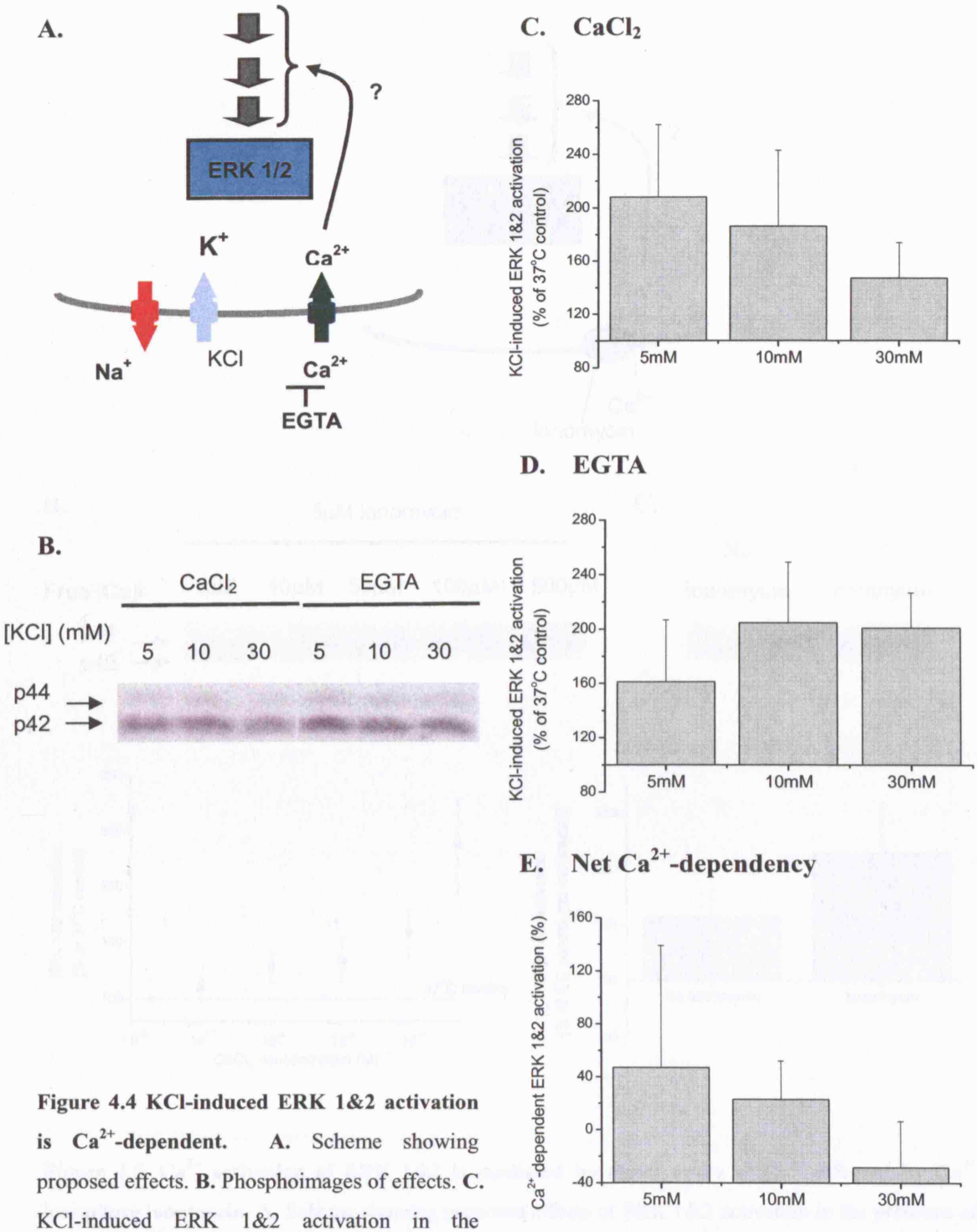



**Figure 4.4 KCl-induced ERK 1&2 activation is $Ca^{2+}$-dependent.** **A.** Scheme showing proposed effects. **B.** Phosphoimages of effects. **C.** KCl-induced ERK 1&2 activation in the presence of $CaCl_2$. **D.** KCl-induced ERK 1&2 activation in the presence of EGTA. **E.** Net $Ca^{2+}$-dependency of ERK 1&2 activation. Synaptosomes (0.3 mg/ml) were incubated with 1mM of $CaCl_2$ or 100µM EGTA added at 3 min, followed by the depolarisation with KCl at 10 min. Experiments carried out with three synaptosomal preparations (n=3).

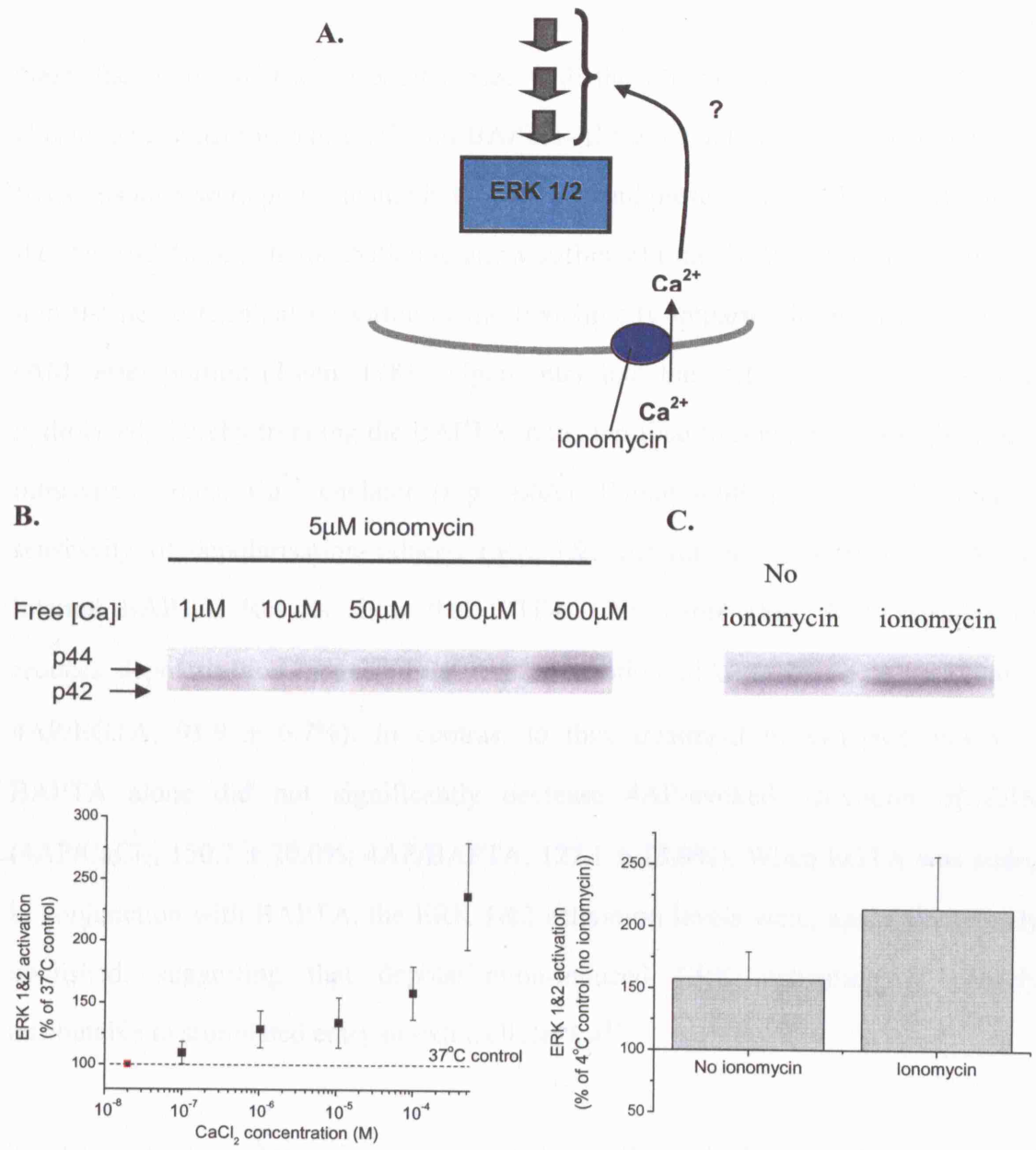



**Figure 4.5. $Ca^{2+}$ activation of ERK 1&2 is mediated by direct entry of $Ca^{2+}$ effected by $Ca^{2+}$ ionophore ionomycin. A.** Scheme showing proposed effects of ERK 1&2 activation in the presence of ionomycin and added buffered free $Ca^{2+}$. **B.** Phosphoimages and quantification of ionomycin-induced phospho-ERK 1&2 levels. Synaptosomes (0.3 mg/ml) were incubated in the presence of 50μM EGTA, added at 3 min, followed by 5μM ionomycin, added at 4 min. Increasing concentrations of buffered free $CaCl_2$ (1μM, 10μM, 50μM, 100μM and 500μM) were added at 10 min. Data represent means ± SEM of experiments carried out with more than ten synaptosomal preparations (n>10). **C.** Phosphoimages and quantification of the effect of ionomycin on basal ERK 1&2 activation on its own. Data represent means ± SEM of experiments carried out with three synaptosomal preparations (n=3).

activation levels (Fig. 4.5C).

Next, the source of $Ca^{2+}$ was addressed with the use of $Ca^{2+}$ chelators, EGTA to chelate extrasynaptosomal $Ca^{2+}$, and BAPTA-AM to chelate intrasynaptosomal $Ca^{2+}$. Synaptosomes were preincubated in the absence and presence of BAPTA-AM prior to the standard 10-minute incubation to allow sufficient time for BAPTA to be delivered into the nerve terminal by virtue of the lipophilicity imparted by its acetoxymethyl (AM) ester portion (Tsien, 1981). Upon entry into the cell, the AM ester bond is hydrolysed, thereby trapping the BAPTA in the terminal to function as a high affinity intrasynaptosomal $Ca^{2+}$ chelator (Fig. 4.6A). Figure 4.6B illustrates the relative sensitivity of depolarisation-induced ERK 1&2 activation to external EGTA and internal BAPTA. Results show that EGTA, like before (Fig. 4.3), significantly reduces depolarisation-induced ERK 1&2 activation (4AP/$CaCl_2$, 150.7 ± 20.0%; 4AP/EGTA, 91.9 ± 6.7%). In contrast to this, treatment of synaptosomes with BAPTA alone did not significantly decrease 4AP-evoked activation of ERK (4AP/$CaCl_2$, 150.7 ± 20.0%; 4AP/BAPTA, 127.1 ± 13.9%). When EGTA was added in conjunction with BAPTA, the ERK 1&2 activation levels were, again, completely abolished, suggesting that depolarisation-induced ERK activation is entirely attributable to stimulated entry of extracellular $Ca^{2+}$.

To determine the effect of removing any intracellular $Ca^{2+}$ released from internal stores on ERK 1&2 activation, synaptosomes were incubated with thapsigargin, an irreversible SERCA pump inhibitor (Treiman et al., 1998; Smith and Reed, 1996) (Fig. 4.7A). Data revealed that 1μM thapsigargin significantly reduced basal ERK 1&2 phosphorylation by 15.0 ± 4.3% (Fig. 4.7B). This reduction of 15% of basal ERK activities appears to correspond with the level of $Ca^{2+}$-dependent activation of basal ERK 1&2 (see Fig. 4.3). This suggests that the $Ca^{2+}$-dependent component of basal ERK 1&2 activity is mainly attributable to intracellular $Ca^{2+}$ released from

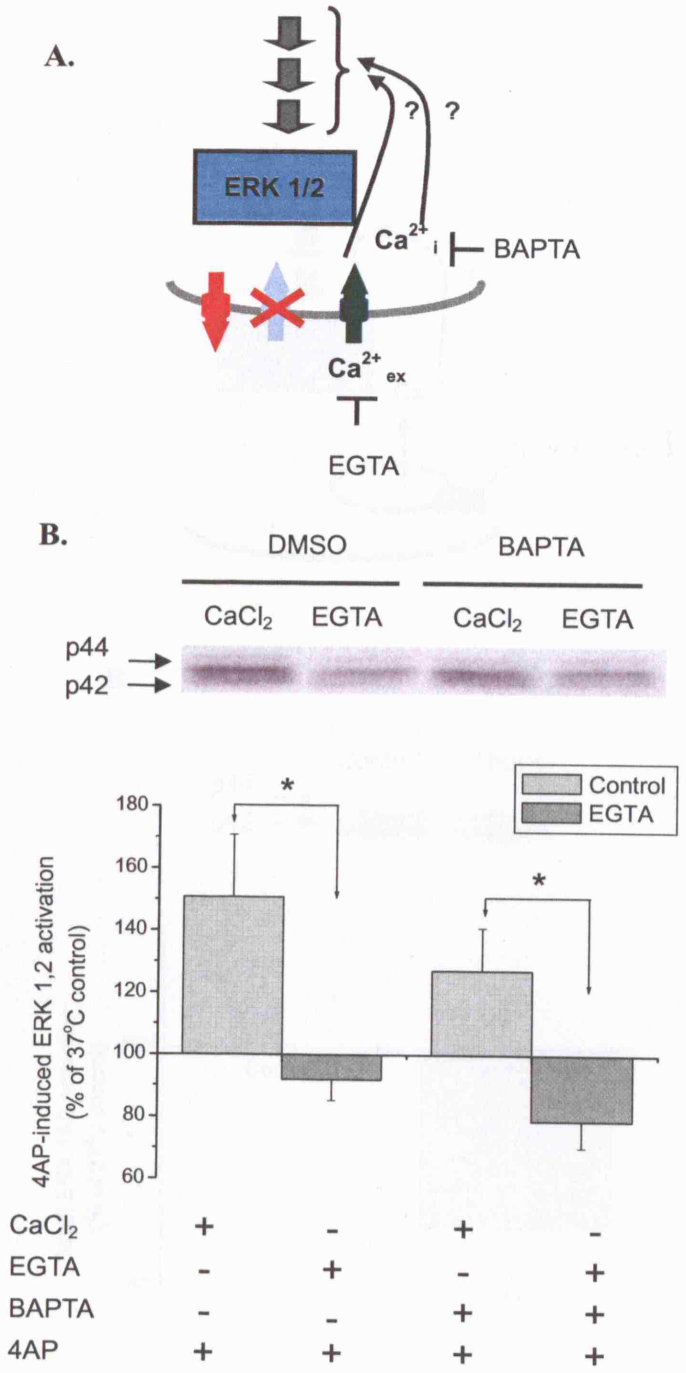



**Figure 4.6. Relative sensitivity of 4AP/depolarisation-induced ERK 1&2 activation to extrasynaptosomal EGTA and intrasynaptosomal BAPTA. A.** Scheme showing proposed effects of $CaCl_2$, EGTA and BAPTA on 4AP-induced activation of ERK. **B.** Phosphoimages and quantification of phospho-ERK 1&2 levels. Synaptosomes (0.3 mg/ml) were preincubated in the absence (Control) and presence of 100μM BAPTA (BAPTA), after which the standard 10-minute incubation protocol was followed as described in the Materials and Methods section. *$p<0.05$ compared to corresponding control with $CaCl_2$ (unpaired Student t-test). Experiments were carried out with seven synaptosomal preparations (n=7).

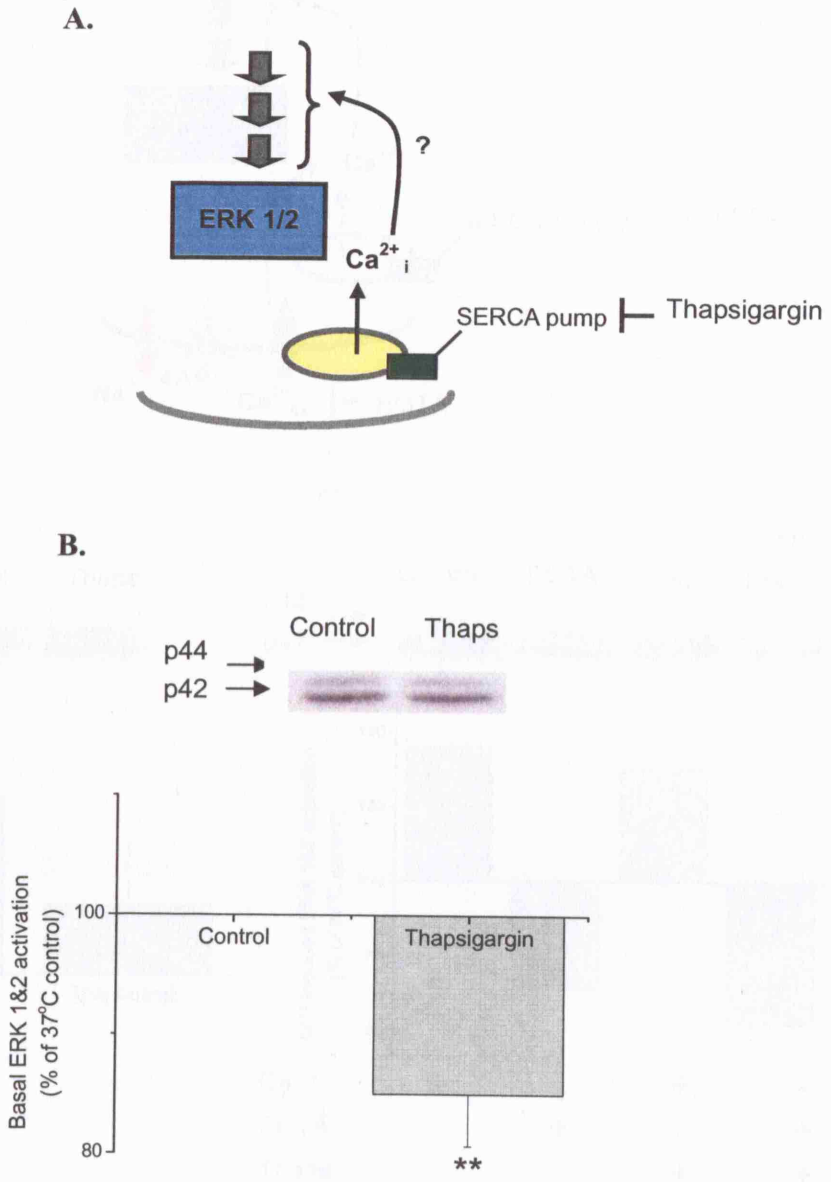



**Figure 4.7. Effect of thapsigargin on basal ERK 1&2 activation.**

**A.** Scheme showing proposed effects of thapsigargin on basal ERK 1&2 activities. **B.** Phosphoimages and quantification of phospho-ERK with and without treatment of thapsigargin. Synaptosomes (0.3 mg/ml) were incubated in the absence (Control) and presence of 1μM thapsigargin (Thapsigargin), added at 7 min. **$p<0.01$ compared to 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with five independent synaptosomal preparations (n=5).

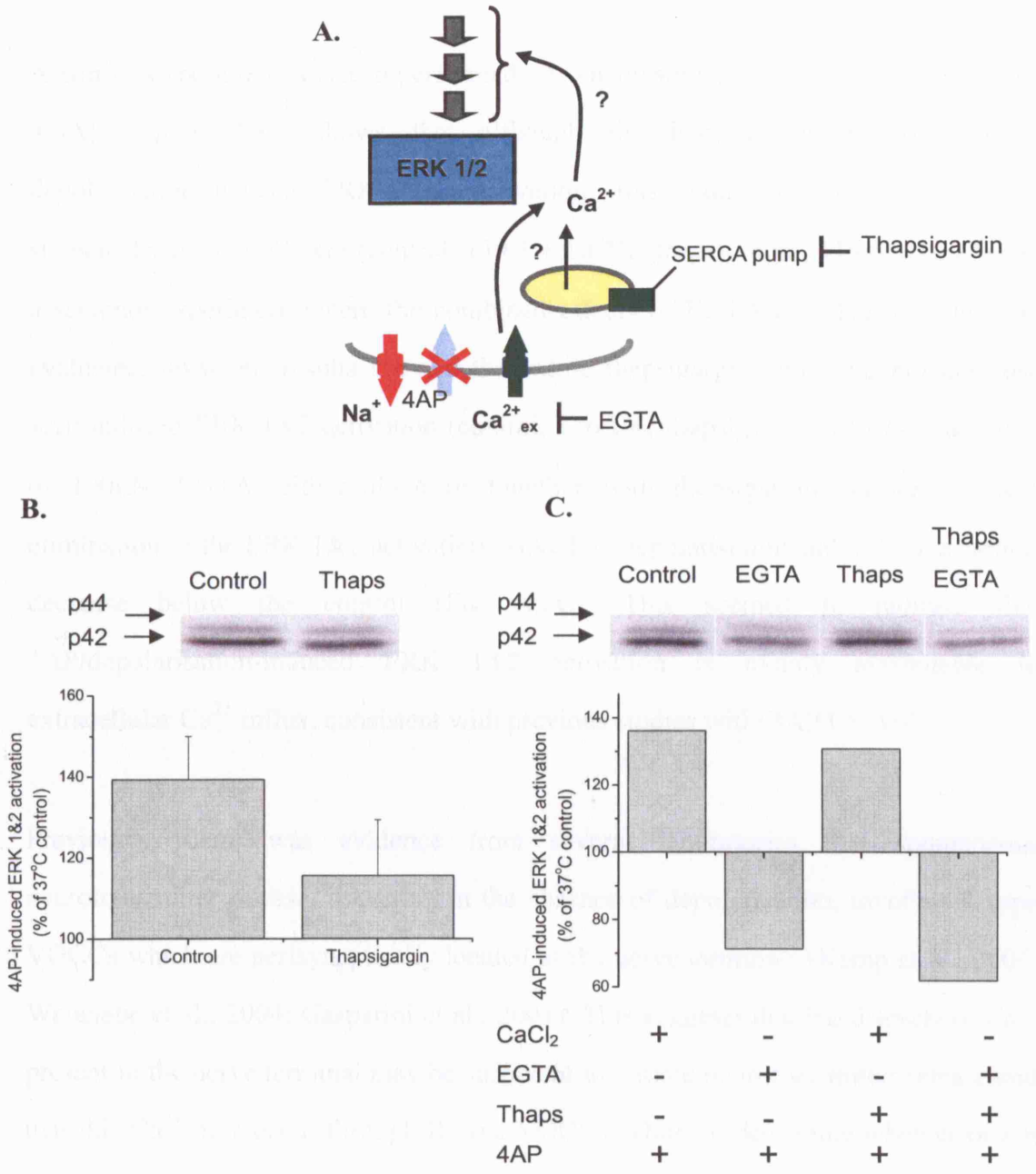



**Figure 4.8. Effect of thapsigargin on 4AP-induced ERK 1&2 activation.**

**A.** Scheme showing proposed effects of thapsigargin on 4AP-induced activation of ERK 1&2. **B.** Phosphoimages and quantification of phospho-ERK 1&2 levels in the absence and presence of thapsigargin. Synaptosomes (0.3 mg/ml) were incubated at 37°C with 1μM thapsigargin in the presence of 1mM of $CaCl_2$ or 100μM EGTA, added at 3 min and 1mM 4AP, added at 10 min. Data represent means ± SEM of experiments carried out with five synaptosomal preparations (n=5). **C.** Phosphoimages and quantification of phospho-ERK 1&2 levels showing the effect of thapsigargin and/or EGTA on 4AP-induced ERK 1&2 activation. The experiment was carried out with one synaptosomal preparation (n=1).

intracellular stores.

A similar experiment was also performed on synaptosomes stimulated with 4AP (Fig. 4.8A). Figure 4.8B shows that although thapsigargin appears to decrease depolarisation-induced ERK 1&2 activation, this reduction was found to be statistically non-significant (control, 139.3 ± 10.7%; thapsigargin, 115.6 ± 13.8%). In a separate experiment where the combined effects of EGTA and thapsigargin were evaluated, however, results showed that while thapsigargin alone did not decrease 4AP-induced ERK 1&2 activation (control, 136.1%; thapsigargin, 130.7%), addition of 100μM EGTA, either alone or together with thapsigargin, showed a clear elimination of the ERK 1&2 activation evoked by depolarisation and indeed, a further decrease below the control (Fig. 4.8C). This seemed to indicate that 4AP/depolarisation-induced ERK 1&2 activation is mainly attributable to extracellular $Ca^{2+}$ influx, consistent with previous studies with BAPTA-AM.

Previously, there was evidence from several laboratories that spontaneous neurotransmitter release, occurring in the absence of depolarisation, involves R-type VGCCs which are perisynaptically located in the nerve terminals (Kamp et al., 2005; Watanabe et al., 2004; Gasparini et al., 2001). This suggests that basal levels of $Ca^{2+}$ present in the nerve terminal may be sufficient to initiate neurotransmitter release and that this $Ca^{2+}$ may occur through R-type VGCCs. Thus, to determine whether or not basal ERK 1&2 activities are supported by $Ca^{2+}$ influx via these R-type VGCCs, the following experiment investigated the effect of blocking R-type VGCCs using synaptosomes prepared from wild-type and mutant mice that had the R-type VGCC genetically ablated (Pereverzev et al., 1998). Both basal and depolarisation-induced ERK 1&2 activation were examined in the presence and absence of 100μM SNX-482 toxin, a synthetic peptide toxin which specifically blocks the $Ca_v2.3$ α1E/R-type VGCCs (Newcomb et al., 1998). Figure 4.9 B indicates that under basal conditions,

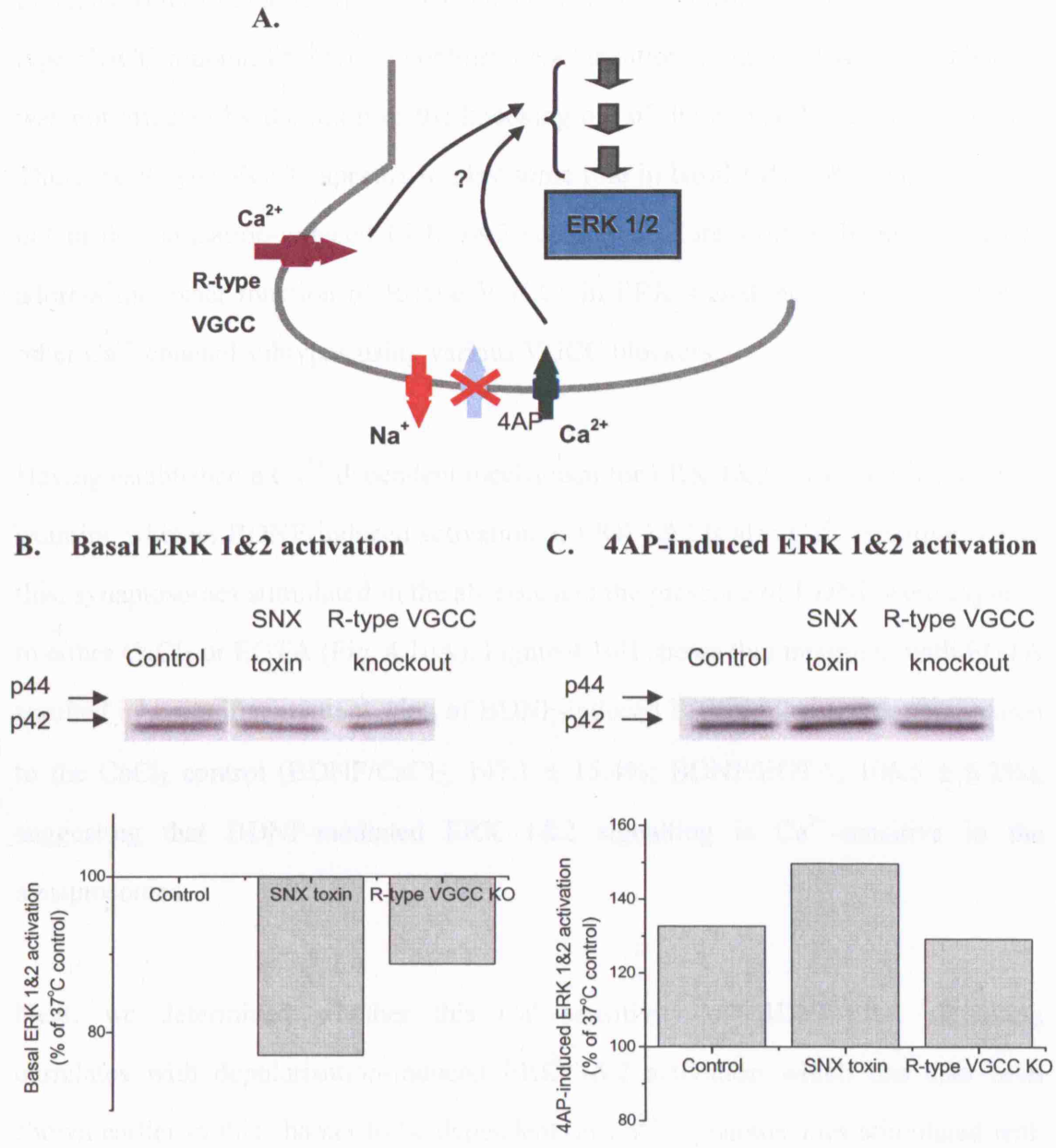



**Figure 4.9. Effect of SNX toxin and R-type VGCC gene mutation on basal and depolarisation-induced ERK 1&2 activation in mice synaptosomes. A.** Scheme showing proposed effects of SNX toxin and R-type VGCC knockout (KO) on ERK 1&2 activation. **B.** Phosphoimages and quantification for basal ERK 1&2 activation. **C.** Phosphoimages and quantification of phospho-ERK for 4AP-induced ERK activation. Synaptosomes (0.3 mg/ml) from wild-type and R-type VGCC knockout mice were incubated with or without 100nM SNX-482 toxin. Depolarisation with 1mM 4AP was carried out at 10 min. The experiment was carried out with one synaptosomal preparation (n=1).

ERK 1&2 activities were attenuated in the wild-type animal treated with the SNX toxin, as well as in the R-type VGCC knockout mice (control, 100%; SNX, 77.0%; R-type VGCC mutant, 88.7%). In contrast, depolarisation-induced ERK 1&2 activation was not affected by the toxin or the knocking out of the R-type VGCCs (Fig. 4.9C). Thus, the R-type VGCC appears to play some role in basal ERK 1&2 activation, but not in depolarisation-induced ERK 1&2 activation. More work will be required to address the exact function of R-type VGCCs in ERK signalling as well as roles of other $Ca^{2+}$ channel subtypes using various VGCC blockers.

Having established a $Ca^{2+}$-dependent mechanism for ERK 1&2 activation, we want to examine whether BDNF-induced activation of ERK 1&2 is also $Ca^{2+}$-sensitive. To do this, synaptosomes stimulated in the absence and the presence of BDNF were exposed to either $CaCl_2$ or EGTA (Fig. 4.10A). Figure 4.10B shows that treatment with EGTA resulted in a significant attenuation of BDNF-induced ERK 1&2 activation compared to the $CaCl_2$ control (BDNF/$CaCl_2$, 147.1 ± 15.4%; BDNF/EGTA, 106.5 ± 5.2%), suggesting that BDNF-mediated ERK 1&2 signalling is $Ca^{2+}$-sensitive in the synaptosomes.

Next, we determined whether this $Ca^{2+}$-sensitivity of BDNF-ERK signalling correlates with depolarisation-induced ERK 1&2 activation which has also been shown earlier in this chapter to be dependent on $Ca^{2+}$. Synaptosomes stimulated with or without BDNF were depolarised with 1mM 4AP (Fig. 4.11A). Figure 4.11B shows that the 4AP-induced and BDNF-facilitated activation of ERK 1&2 were not additive (4AP, 154.89 ± 8.98%; 4AP/BDNF, 151.74 ± 6.63%). Furthermore, the ionomcyin-induced increase of ERK 1&2 activation was not affected by treatment with BDNF (Fig. 4.12A and 4.12B: control, 178.53 ± 23.11%; BDNF, 158.43 ± 32.47%). These results suggest that the BDNF and depolarisation/$Ca^{2+}$-induced pathway to ERK 1&2 are two independent inputs that feed into a common ERK 1&2 signalling cascade.

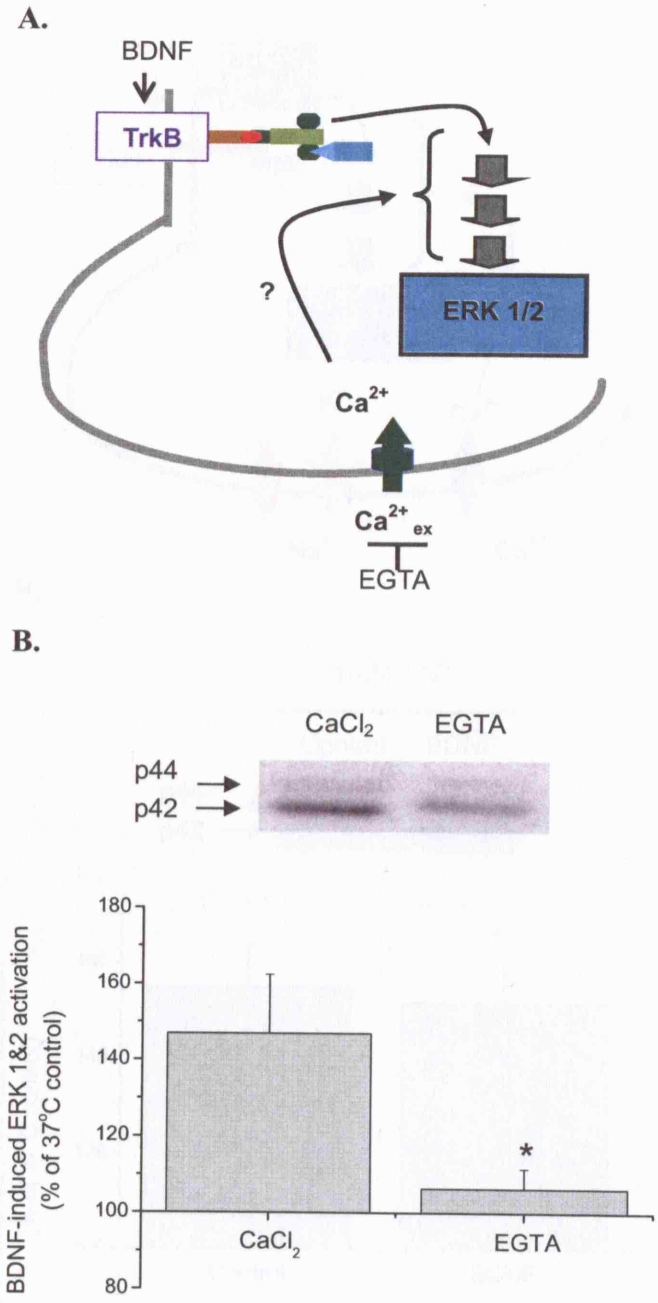



**Figure 4.10. BDNF-induced ERK 1&2 activation is $Ca^{2+}$-sensitive.**

**A.** Scheme showing proposed effects of $CaCl_2$ and EGTA on neurotrophin-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of BDNF-induced ERK levels following treatment in the presence of $CaCl_2$ or EGTA. Synaptosomes (0.3 mg/ml) were incubated in the presence of 200ng/ml BDNF with the addition of 1mM of $CaCl_2$ or 100μM EGTA at 3 min. *$p<0.05$ compared to 37°C $CaCl_2$ control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with five independent synaptosomal preparations (n=5).

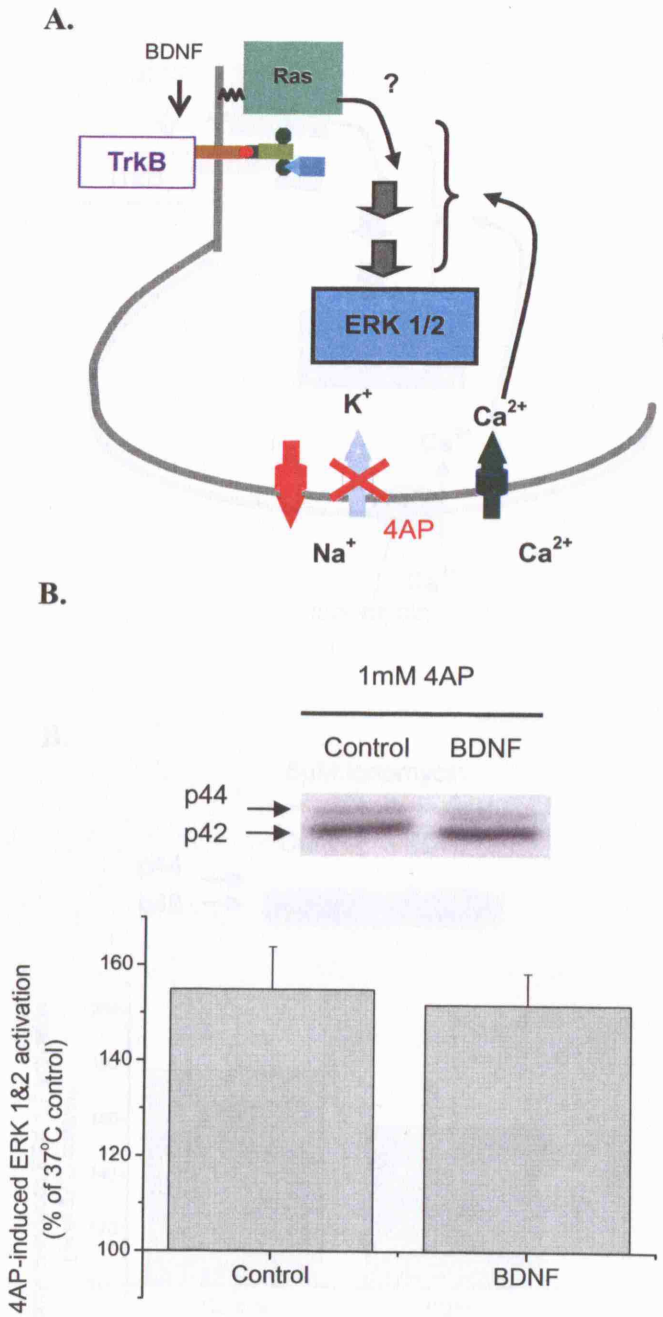



**Figure 4.11. Effect of Trk B agonist BDNF on 4AP-induced ERK 1&2 activation.**

**A.** Scheme showing proposed effects of BDNF on 4AP-induced activation of ERK. **B.** Phosphoimages and quantification of 4AP-induced phospho-ERK levels with or without BDNF addition. Synaptosomes (0.3 mg/ml) were incubated in the absence (Control) and presence of 200ng/ml BDNF (BDNF) before depolarisation with 1mM 4AP at 10 min. Data represent means ± SEM of experiments carried out with three independent synaptosomal preparations (n=3).

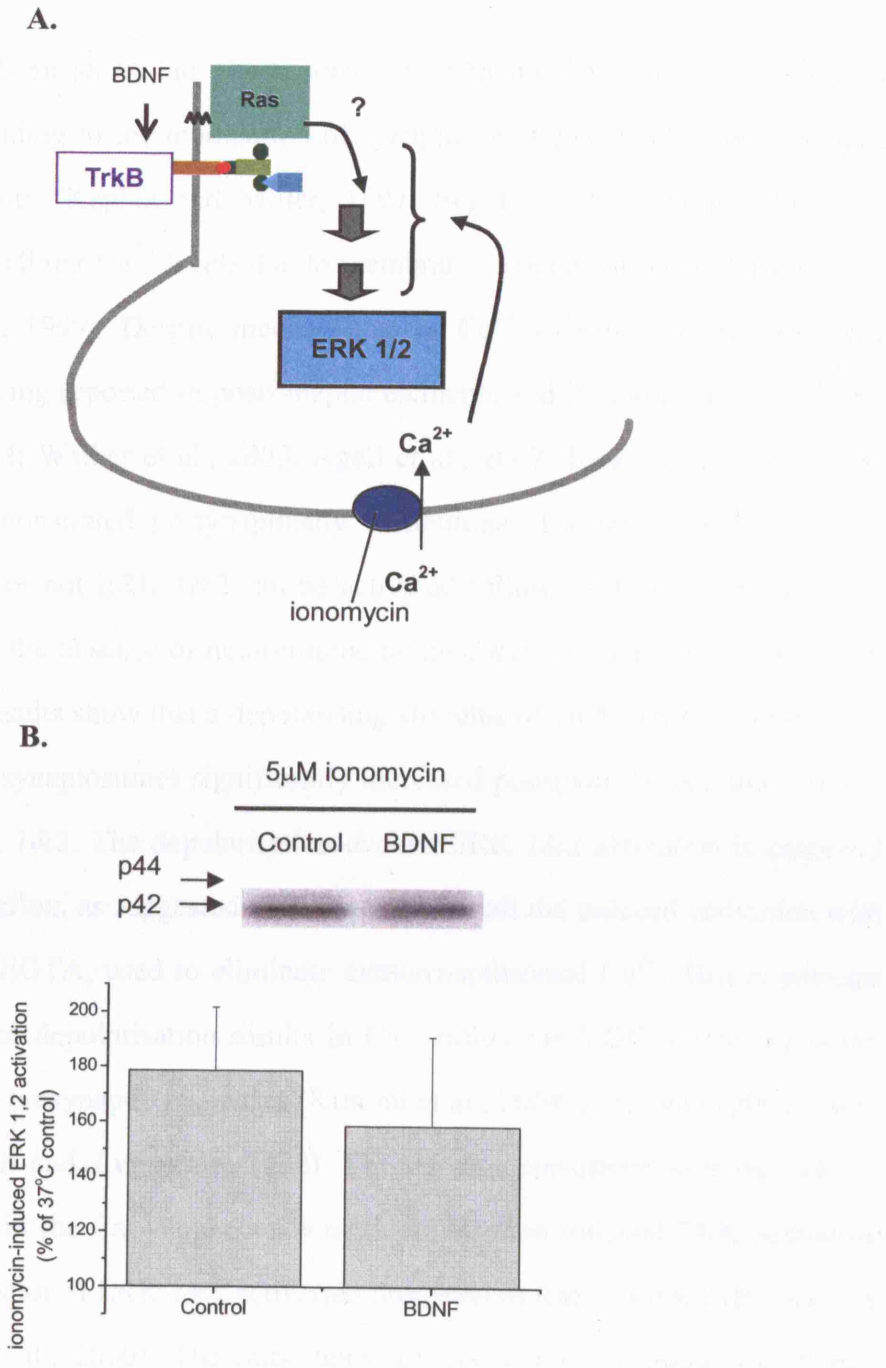



**Figure 4.12. Effect of BDNF on $Ca^{2+}$-dependent ERK 1&2 activation effected by ionomycin.**

**A.** Scheme showing proposed effects of BDNF on ionomycin-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of ionomycin-induced phospho-ERK levels with and without BDNF treatment. Synaptosomes (0.3 mg/ml) incubated in the absence (Control) and presence of 200ng/ml BDNF (BDNF) with 50μM EGTA added at 3 min, 5μM ionomycin at 4 min followed by 500μM buffered free $CaCl_2$ at 10 min. Data represent means ± SEM of experiments carried out with five independent synaptosomal preparations (n=5).

## 4.4. Discussion

ERK 1&2 have been shown to play a major role in mediating trophic effects of several stimuli leading to the modulation of synaptic plasticity. Such stimuli include neurotrophic factors (Kaplan and Miller, 1997; Segal and Greenberg, 1996) and increases in intracellular $Ca^{2+}$ levels due to membrane depolarisation (Baldassa et al., 2003; Egea et al., 1999). Despite mechanisms for $Ca^{2+}$ activation of Ras-mediated ERK signalling being reported in postsynaptic elements and in non-neuronal systems (Aspenstrom, 2004; Walker et al., 2003; Agell et al., 2002; Rosen et al., 1994), this has not been demonstrated presynaptically in neurons. The work in this chapter explored whether or not ERK 1&2 can be activated following depolarisation and/or by $Ca^{2+}$ signals in the absence of neurotrophic factors, and the mechanisms by which this occurs. The results show that a depolarising stimulus of 1mM 4AP or 30mM KCl to cerebrocortical synaptosomes significantly increased phosphorylation, and thereby, activation of ERK 1&2. The depolarisation-evoked ERK 1&2 activation is preceded by external $Ca^{2+}$ influx, as suggested by suppression of all the induced activation with the $Ca^{2+}$ chelator EGTA, used to eliminate extrasynaptosomal $Ca^{2+}$. This is perhaps not surprising since depolarisation results in $Ca^{2+}$ influx via VGCCs into the nerve terminal to initiate presynaptic responses (Kuromi et al., 2004; Augustine, 2001; Sihra et al., 1993; Smith and Augustine, 1988). This is also consistent with the role of extracellular $Ca^{2+}$ in the $Ca^{2+}$-dependency of depolarisation-induced ERK activation as a common regulator of ERK 1&2 activation upstream of Ras and Rap1 (Baldassa et al., 2003; Egea et al., 2000). The importance of external $Ca^{2+}$ influx was further highlighted by the fact that intrasynaptosomal $Ca^{2+}$ appears to play little role in the depolarisation-induced activation of ERK 1&2. This was evinced by the insignificant effects of both intrasynaptosomal $Ca^{2+}$ chelator BAPTA-AM and SERCA pump inhibitor thapsigargin on the 4AP-induced stimulation of ERK 1&2. Furthermore, corroboration for $Ca^{2+}$-dependency of both basal and depolarisation-induced ERK 1&2 was obtained by using the $Ca^{2+}$ ionophore, ionomycin, which allows the titration

of $Ca^{2+}$ into the nerve terminal independently of VGCCs activation. Results showed that ERK 1&2 activation was enhanced in the presence of buffered free $Ca^{2+}$, added in combination with ionomycin, suggesting that $Ca^{2+}$ can induce ERK 1&2 activation.

Interestingly, $Ca^{2+}$-dependency was also clearly seen in basal ERK 1&2 activities, where approximately 20% of the basal activation was attenuated with EGTA. This suggests that a significant proportion of the basal activity is $Ca^{2+}$-independent. With regard to the small but significant $Ca^{2+}$-dependent component of basal ERK activity, the question arises as to the source of the $Ca^{2+}$ supporting this activation. One possibility is that this may be attributable to intracellular $Ca^{2+}$ released from internal $Ca^{2+}$ stores. The evidence for this stems from the ability of thapsigargin to significantly reduce the $Ca^{2+}$-dependent component of the 37°C/basal activation of ERK 1&2. This finding is in agreement with the role of intracellular $Ca^{2+}$ playing a part in ERK 1&2 activation as previously suggested (Aspenstrom, 2004; Walker et al., 2003; Baldassa et al., 2003; Agell et al., 2002; Crossthwaite et al., 2002; Egea et al., 2000). There is also evidence that $Ca^{2+}$-induced intracellular $Ca^{2+}$ released from stores contributes to mediating short-term plasticity and spontaneous transmitter release (Emptage et al., 2001).

It is evident from the foregoing discussion relating to the thapsigargin data that basal ERK 1&2 activation is primarily due to intracellular $Ca^{2+}$. Interestingly, blockade of R-type VGCCs using the R-type VGCC knockout mice and the inhibitor of this channel was able to produce a similar inhibition on basal ERK 1&2 activation. The question is, then: is the basal ERK 1&2 activation dependent on both intracellular $Ca^{2+}$ released from stores and external $Ca^{2+}$ influx through the R-type VGCCs? One possibility is that $Ca^{2+}$ influx through R-type VGCCs may initiate a $Ca^{2+}$-induced $Ca^{2+}$ leakage from intracellular stores, and therefore, the use of thapsigargin is then capable of removing all of the $Ca^{2+}$ released, producing an inhibition of basal ERK 1&2 activities similar to that obtained by blocking R-type VGCC. Notably, with regard to

the R-type channel, there is evidence to suggest that R-type VGCCs are involved in spontaneous neurotransmitter release as well as neuromodulation (Kamp et al., 2005; Watanabe et al., 2004; Gasparini et al., 2001). Thus, it is likely that extracellular $Ca^{2+}$ influx through these R-type channels contribute to the basal activation of ERK 1&2. The effect of thapsigargin and R-type channel blockade in basal ERK 1&2 activation, however, contrasts with the behaviour of depolarisation-induced ERK 1&2 activation, where the use of R-type channel toxin and R-type VGCC mutation has a non-significant effect. These data suggest that depolarisation may activate $Ca^{2+}$ influx via the N and P/Q-type VGCCs (that are insensitive to the R-type toxin) to take over function for the subsequent activation of ERK 1&2. However, further work has to be done to address this directly.

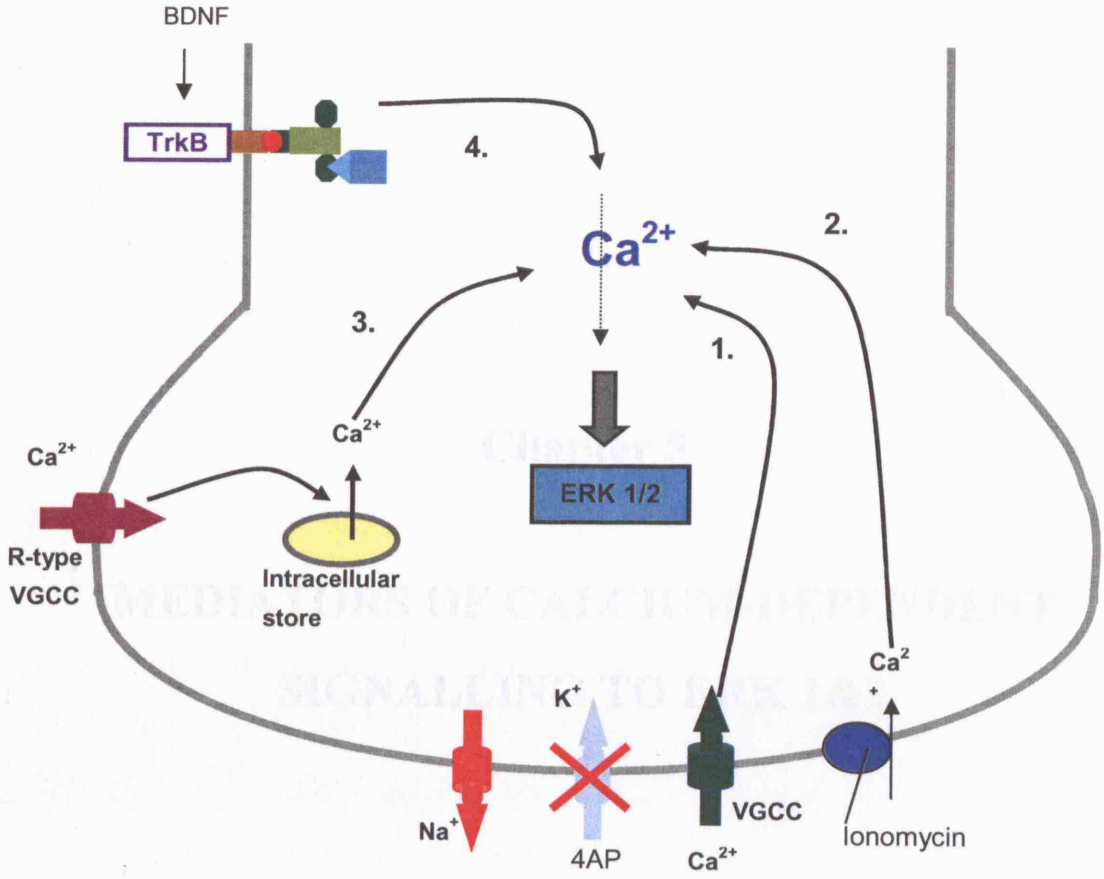
Thus far, we have shown that activation of ERK 1&2 is contingent on $Ca^{2+}$, but what is the target of this $Ca^{2+}$ signal? Two possibilities can be presented: i) $Ca^{2+}$ acts on upstream regulators of ERK 1&2; ii) $Ca^{2+}$ acts on the MAP kinase itself. While there is no evidence for direct activation of ERK 1&2 by $Ca^{2+}$, there is evidence to suggest that $Ca^{2+}$ is capable of modulating the Ras regulators such as Ras-GEF and Ras-GAPs (Aspenstrom, 2004; Walker et al., 2003; Bos, 1998; Ebinu et al., 1998; Farnsworth et al., 1995). As well as modulation at the level of Ras activation (Finkbeiner and Greenberg, 1996), $Ca^{2+}$-regulation of ERK signalling has also been shown to be exerted at sites downstream of Ras such as at the level of Raf-1 (Egea et al., 2000; Egea et al., 1999). Altogether, our data and previous evidence point to the existence of a $Ca^{2+}$-dependent, neurotrophin-independent pathway for ERK 1&2 activation where presynaptic ERK 1&2 activation can be regulated by both depolarisation-evoked $Ca^{2+}$ signals and/or changes in intracellular $Ca^{2+}$ levels. Moreover, this neurotrophin-independent signalling was confirmed by our observation that BDNF-mediated ERK 1&2 signalling and the depolarisation-induced increase $Ca^{2+}$-dependent ERK 1&2 activation were not additive. This occlusion by BDNF of depolarisation/$Ca^{2+}$-stimulated ERK 1&2 activation suggests that the BDNF-mediated pathway and the

depolarisation/$Ca^{2+}$-induced ERK 1&2 activation pathway are two separate inputs that feed into a single ERK 1&2 signalling cascade. This is consistent with studies that concluded the independent mechanisms by which depolarisation or $Ca^{2+}$ induce ERK signalling from that of the Trk-mediated pathway (Baldassa et al., 2003; Egea et al., 1999; Rosen et al., 1994).

Interestingly, the role of $Ca^{2+}$ in ERK 1&2 activation was also demonstrated in BDNF-evoked ERK 1&2 activation. We show that facilitation of ERK 1&2 activation by BDNF was attenuated with the addition of the $Ca^{2+}$ chelator EGTA. This $Ca^{2+}$-sensitivity may be due to two possibilities: i) the ability of BDNF to modulate $Ca^{2+}$ influx via VGCCs (Baldelli et al., 2000; Baldelli et al., 1999), thereby, increasing $Ca^{2+}$ levels for the subsequent activation of ERK 1&2; or ii) $Ca^{2+}$ may have a permissive role in BDNF stimulated conditions. Given that BDNF, itself, does not increase intracellular $Ca^{2+}$ levels in synaptosomes (see chapter 7), the role of $Ca^{2+}$ in providing a permissive pathway for BDNF signalling seems likely. This finding seems to agree with previous studies implicating $Ca^{2+}$ in the BDNF-induced ERK 1&2 signalling where the presence of $Ca^{2+}$ is required for the BDNF-mediated effects to occur (Goggi et al., 2002; Kang and Schuman, 2000; Stoop and Poo, 1996). In addition, the requirement of $Ca^{2+}$ for neurotrophic effects has been demonstrated in spinal motor neurons (Meyer-Franke et al., 1998). Under these conditions, BDNF has been shown to be more readily released following strong depolarisation. In addition, there is evidence that $Ca^{2+}$ influx causes the upregulation of Trk receptor insertion into the membrane after high frequency stimulation (Meyer-Franke et al., 1998). Given that the BDNF used in our experiments are exogenously applied, it is unlikely that the $Ca^{2+}$ requirement of BDNF-mediated signalling to ERK 1&2 is due to an increase BDNF release. However, whether or not the $Ca^{2+}$ is required for the functionality of Trk receptor insertion and the subsequent TrkB-ERK signalling to occur is yet to be examined, although this is unlikely to be addressable with our current experimental paradigm in synaptosomes. Another possibility is that the presence of $Ca^{2+}$ may

enable the BDNF-mediated Ras activation to take place more efficiently. In this regard, it is possible that $Ca^{2+}$ may result in the activation of presynaptic CaMKII which has been shown previously to mediate inhibition of Ras-GAP (Oh et al., 2004; Bos, 1998; Chen et al., 1998). Under such conditions where Ras inactivation is constitutively inhibited, ERK 1&2 activation by BDNF may be achieved more effectively on increasing the activities of Ras-GEF or GRF. Further experiments are warranted to elucidate this point. Taken together, our data seem to suggest that BDNF may be intrinsically linked to $Ca^{2+}$/neuronal activity in order to mediate downstream ERK 1&2 signalling.

In conclusion, the ERK signal transduction pathway in nerve terminals can be activated through a $Ca^{2+}$-dependent mechanism, either by membrane depolarisation or by direct $Ca^{2+}$ entry effected by ionophore, independently of any neurotrophic input such as BDNF. At the same time, BDNF itself displays $Ca^{2+}$-sensitivity in the absence of any stimulated influx of $Ca^{2+}$. Thus, depolarisation/$Ca^{2+}$-induced and neurotrophic-mediated pathways appear to be two independent inputs that converge to effect ERK 1&2 activation **(Schematic 4.4)**.




**Schematic 4.4. The potential routes that feed into $Ca^{2+}$-dependent activation of ERK 1&2.** 1.) ERK 1&2 activation is induced by external $Ca^{2+}$ influx following depolarisation of plasma membrane by 4AP or KCl; 2.) $Ca^{2+}$-dependent activation of ERK 1&2 is effected with the $Ca^{2+}$ ionophore ionomycin; 3.) Basal ERK 1&2 activation was contributed by intracellular $Ca^{2+}$ released from store, possibly including $Ca^{2+}$-induced $Ca^{2+}$ release following R-type VGCC activation; 4.) $Ca^{2+}$ provides a permissive role for BDNF-mediated ERK 1&2 activation. This may possibly be achieved through the CaMKII-inhibition of Ras-GAP, thus enabling BDNF-signalling to ERK 1&2 to occur more effectively with increasing Ras-GEF or GRF activities. BDNF-mediated activation of ERK is a separate input from depolarisation-induced signalling to ERK 1&2.

# Chapter 5

# MEDIATORS OF CALCIUM-DEPENDENT SIGNALLING TO ERK 1&2

## 5.1. Introduction

It has long been established that $Ca^{2+}$ functions as an important stimulator of a number of protein kinase cascades. In the foregoing chapter, we have provided evidence of a $Ca^{2+}$-dependent cascade leading to ERK 1&2 activation in the cerebrocortical nerve terminals. Thus, the immediate question arising is: what is the basis of this $Ca^{2+}$-dependency? In so far as there is no evidence for a direct effect of $Ca^{2+}$ on the activation of ERK 1&2, how then could this $Ca^{2+}$ signal become translated to downstream ERK 1&2 activation and what are the intermediate steps that allow ERK to be regulated by the intrasynaptosomal levels of $Ca^{2+}$?

Previous studies have implicated calmodulin (CaM) as a prime mediator of $Ca^{2+}$-dependent signalling (Agell et al., 2002; Means, 2000; Klee et al., 1998). CaM is a $Ca^{2+}$-sensing protein that is known to transduce intracellular $Ca^{2+}$ signal for many cellular responses. By binding to $Ca^{2+}$, a conformational change in CaM is initiated to allow CaM to bind to its target effectors. Indeed, several reports have shown that CaM is involved in the regulation of signalling leading to the activation of ERK 1&2 (Agell et al., 2002; Egea et al., 2000; Egea et al., 1999). Studies have shown that CaM is required in neuronal survival, where BDNF signalling can be inhibited by W13, a CaM inhibitor (Egea et al., 2001). Experiments with cultured striatal neurons have also suggested $Ca^{2+}$ and CaM as important regulators in the activation of ERK 1&2 following NMDA receptor activation (Perkinton et al., 2002). In addition, CaM has been implicated in mediating ERK activation induced either by depolarisation (Egea et al., 1999) or by ionomycin (Quinn et al., 2002) in PC12 cells. Presynaptically, while the role of CaM in $Ca^{2+}$-induced neurotransmitter release from synaptosomes has also been demonstrated using various CaM antibodies and antagonists (Hens et al., 1996; Augustine et al., 1987), whether the molecule is involved in ERK pathway has not been considered.

Downstream of the signalling cascade, one of the target proteins activated by $Ca^{2+}$ and CaM is $Ca^{2+}$/CaM protein kinase II (CaMKII), a multifunctional serine/threonine kinase found localised at high concentrations in both presynaptic and postsynaptic compartments, and thought to be instrumental in a number of synaptic plasticity (Griffith, 2004; Schulman, 2004; Elgersma et al., 2002; Soderling et al., 2001; Glazewski et al., 2000; Kennedy, 1998; Giese et al., 1998; Nayak et al., 1996; Liu and Jones, 1996). Upon binding of $Ca^{2+}$ and CaM, the autoinhibitory subunit of CaMKII is unbound from the substrate-binding pocket by an intramolecular conformational change so that the enzyme is activated (Thiel et al., 1988; Lai et al., 1986). As studies have demonstrated that CaMKII can associate with SVs through its catalytic domain (Benfenati et al., 1996; Benfenati et al., 1992b) as well as acting as an upstream regulator for a number of kinases, phosphatases and synaptic proteins such as synapsin I (Colbran and Brown, 2004; Hudmon and Schulman, 2002; Nayak et al., 1996; Benfenati et al., 1996), this kinase appears to play an essential role in mediating presynaptic $Ca^{2+}$ and CaM signals for subsequent cellular response. This is supported by evidence that CaMKII can increase neurotransmitter release from both the squid giant synapse (Llinas et al., 1991; Lin et al., 1990) and synaptosomes from rat brain (Sihra and Pearson, 1995; Nichols et al., 1990). This enhancement in neurotransmitter release has been shown to be associated with an increase in phosphorylation on synapsin phosphosites 2&3 (Sihra and Pearson, 1995; Nichols et al., 1990). Consistent with this, the role of CaMKII in SV recruitment for exocytosis has also been highlighted in cell biological studies where the rate of synapsin I dispersion and SV pool turnover is controlled by CaMKII phosphorylation of synapsin Ia (Chi et al., 2003). In the vascular smooth muscle, $Ca^{2+}$-dependent ERK 1&2 activation has been shown to be mediated by CaMKII, as suggested by the sensitivity to the CaMKII inhibitor, KN93 (Ginnan and Singer, 2002; Abraham et al., 1997). Furthermore, KN93 has been shown to block glutamate-induced mini-excitatory post-synaptic currents (mEPSCs) and presynaptic functions in hippocampal pyramidal neurons (Ninan and Arancio, 2004).

Apart from CaMKII, studies have also pointed to Ras as a target component of the ERK pathway downstream of $Ca^{2+}$/CaM activation that can be affected by the binding of $Ca^{2+}$ signal-transducing proteins (Aspenstrom, 2004; Walker et al., 2003; Bos, 1998). Reports have shown that Ras can be activated by Ras-GRF that contains a $Ca^{2+}$/CaM-binding domain (Bos, 1998; Farnsworth et al., 1995). Thus, upon $Ca^{2+}$/CaM binding, CaM-Ras-GRF can activate Ras by inducing Ras-GTP exchange. Furthermore, CaM can indirectly regulate SynGAP, a GAP for Ras, through the stimulation of CaMKII-mediated phosphorylation, and in so doing, decreases the SynGAP activity to cause an increase in Ras/ERK activation in a CaM-dependent manner (Oh et al., 2004; Bos, 1998; Chen et al., 1998).

Previously, studies have suggested PI3K as a possible mediator for $Ca^{2+}$-dependent ERK 1&2 activation. Data have shown that apart from activation following RTK (Katso et al., 2001) and GPCR stimulation (Murga et al., 2000; Lopez-Ilasaca et al., 1997), PI3K could be activated in response to increases in cytosolic $Ca^{2+}$ levels. This is evident as PI3K has been shown to be activated by a direct association of $Ca^{2+}$/CaM with the SH2 domains of the regulatory p85 subunit of the kinase (Joyal et al., 1997). In addition, $Ca^{2+}$ may induce PI3K function indirectly through $Ca^{2+}$-induced activation of Ras (Rommel et al., 1999; Downward, 1998; Rodriguez-Viciana et al., 1994). Furthermore, there is evidence that PI3K is involved in mediating ERK 1&2 signalling cascade in a number of systems (Crossthwaite et al., 2002; Perkinton et al., 2002; Chandler et al., 2001; Murga et al., 2000). In some cases, PI3K may regulate activation of ERK 1&2 through its protein kinase activity to regulate activities of Raf kinases or MEK (Bondeva et al., 1998; Hu et al., 1995). PI3K may also enable activation of its downstream target Akt or PKB (protein kinase B) to cause phosphorylation of transcription factors such as CREB (Perkinton et al., 2002; Bonni et al., 1999). This phosphorylation of CREB may then upregulate pro-survival factors for the activation of ERK 1&2. Taken together, this evidence suggests multiple

mechanisms by which PI3K may act to serve as a mediator for the $Ca^{2+}$-dependent signalling to ERK 1&2.

On the contrary to the positive regulation of $Ca^{2+}$-dependent ERK 1&2 through the activation of CaMKII, Ras and PI3K, recent reports have suggested cyclin-dependent kinase 5 (cdk5) might also impinge on the ERK 1&2 signalling in an inhibitory fashion (Kesavapany et al., 2004; Sharma et al., 2002). Cdk5 is a proline-directed serine/threonine kinase that can phosphorylate diverse substrates and has multifunctional roles (Smith et al., 2001; Dhavan and Tsai, 2001; Giese et al., 2005). Previous studies have shown that cdk5 can be regulated by the calpain-mediated $Ca^{2+}$-dependent proteolysis of its p35 activator to the more potent p25 activator (Hisanaga and Saito, 2003; Lee et al., 2000b; Tsai et al., 1994). In cerebellar synaptosomes, studies have revealed increased levels of p25 following depolarisation-induced $Ca^{2+}$ influx (Vairea and Sihra, personal communication). Upon activation, cdk5 has been demonstrated to phosphorylate MEK 1&2 on specific threonine residue ($Thr^{286}$) to downregulate their activities, and thereby, decrease the activation of ERK 1&2 (Kesavapany et al., 2004; Sharma et al., 2002). Furthermore, p35/cdk5 has also been shown to associate with, and thus, phosphorylate RasGRF2, resulting in the downregulation of Rac-dependent ERK 1&2 activities both *in vitro* and *in vivo* (Kesavapany et al., 2004). This is further supported by studies of cdk5 knockout mice which showed higher ERK 1&2 activities as well as increased neurofilaments phosphorylation, suggesting that the loss of cdk5 activity can lead to an up-regulation of ERK 1&2 activity (Hallows et al., 2003; Ohshima et al., 1996). In addition, there is evidence that the activation of Ras/ERK 1&2 signalling by the binding of neurotrophin NGF can induce the transcriptional upregulation of p35 (Weishaupt et al., 2003; Harada et al., 2001). This suggests a negative feedback mechanism for the reciprocal control between cdk5 and ERK 1&2 where cdk5 might decrease the phosphorylation of ERK 1&2 by inhibiting MEK 1&2 and eventually reduce the expression of the cdk5 activator p35.

The foregoing evidence suggests a number of mediators for $Ca^{2+}$-dependent ERK 1&2 signalling, however, none of these studies examined the presynaptic nerve terminal. This chapter sought to elucidate potential upstream mediators/regulators of the $Ca^{2+}$-dependent signalling of ERK 1&2 activation in synaptosomes. Using inhibitors of CaM and CaMKII, their involvement in basal, depolarisation-induced and BDNF-mediated activation of ERK 1&2 was explored. We also aimed to identify the importance of Ras in this $Ca^{2+}$-dependent pathway. In addition, using a specific inhibitor of PI3K, we sought to examine the function of PI3K in mediating $Ca^{2+}$-dependent activation of ERK 1&2. Finally, the role of cdk5 in ERK 1&2 signalling was investigated to determine whether or not it is involved in modulating basal, depolarisation-induced ERK 1&2 activities or neurotrophin-mediated signalling in the presynaptic compartment.

## 5.2. Materials and Methods

### 5.2.1. Synaptosomal preparation

Synaptosomes were prepared as in section 2.1.1.

### 5.2.2. Glutamate release experiment

Glutamate release assay was followed as described in section 2.2.1. Synaptosomes were incubated in HBM containing 1mg/ml BSA with LY29004 (100μM) added at the start of the incubation. $CaCl_2$ (1mM) was added at 3 min and depolarisation with 4AP (1mM) was achieved at 10 min.

### 5.2.3. Standard incubation protocol for ERK 1&2 activation/phosphorylation

Synaptosomal pellets were resuspended in HBM containing 1mg/ml BSA and incubated for a total of 10 minutes as described in section 2.4.1. W7 (50μM), KN93 (5μM), lovastatin (50μM) or roscovitine (5μM) was added at the start of the incubation before the addition of 1mM $CaCl_2$ or 100μM EGTA at 3 min. Depolarisation was achieved with 1mM 4AP at 10 min and reactions were terminated with the addition of sample buffer 1 min after the secretagogue.

### 5.2.4. Preincubation protocol

Synaptosomes were preincubated at 37°C in HBM containing 1mg/ml BSA in the presence of 1mM $CaCl_2$ as described in section 2.4.2. Vehicle/DMSO (1%), KN93 (5μM) or lovastatin (10μM) was added at 10 min and synaptosomes further incubated for 20 minutes. Synaptosomes were then centrifuged at 10,000xg and the supernatant was discarded. The synaptosomal pellet was cooled back to 4°C for 5 min before following the standard incubation protocol as described in section 5.2.3.

### 5.2.5. Protocol measuring $Ca^{2+}$-dependency of ERK 1&2 with ionomycin and

**$Ca^{2+}$-buffers**

Synaptosomes were incubated as indicated in section 2.4.3 in the HBM buffer containing 1mg/ml BSA. EGTA (50μM) was added 3 min followed by the addition of ionomycin (5μM) at 4 min and calcium (500μM final free concentration) was added at 10 min before reaction was stopped with the addition of sample buffer. Drugs of interest were added at the times shown in the figure legends.

### 5.2.6. SDS-PAGE and Immunoblotting

Samples were separated on SDS-PAGE and subjected to immunoblotting as described in detail in sections 2.4.5 and 2.4.6.

### 5.2.7. Statistical analysis

All statistical analysis was carried out using Student's paired t-test.

### 5.2.8. Reagents

A W7 stock of 1mM was made in water and used at a final concentration of 50μM.

A stock solution of KN93 (5mM) was obtained with DMSO before a 500μM working solution was made with HBM. KN93 was used at a final concentration of 5μM.

A 10mM stock solution of lovastatin was made using DMSO and then diluted to a 1mM working solution in HBM. Lovastatin was used at final concentration of 10μM.

LY29004 was made up to a stock solution of 100mM using DMSO before diluting to 10mM working solution in HBM and used at 100μM as a final concentration.

A 5mM stock solution of roscovitine was made with DMSO before diluting to 500μM using HBM. Roscovitine was used at a final concentration of 5μM.

## 5.3. Results

To examine the role of CaM in basal and depolarisation-induced ERK 1&2 activation, synaptosomes were treated with W7, a cell-permeant CaM antagonist (Hidaka and Ishikawa, 1992), prior to depolarisation with 4AP (Fig. 5.1A). W7 was used at a final concentration of 50μM to minimise the non-specific effects previously shown to be produced when used at higher concentrations (Hens et al., 1996). Figure 5.1B shows that the addition of W7 appeared to reduce basal ERK 1&2 activation although this effect failed to reach statistical significance (W7, 95.9 ± 3.6% compared to 100% 37°C control). More significantly, activation of ERK 1&2 evoked by depolarisation with 1mM 4AP was totally abolished by W7 (4AP, 140.1 ± 13.8%; 4AP/W7, 91.2 ± 9.2%), suggesting an essential function of CaM in mediating the $Ca^{2+}$-dependent signals of ERK signalling during depolarisation (Fig. 5.1C).

To confirm the involvement of CaM in the $Ca^{2+}$-dependent ERK 1&2 cascade and to further explore the $Ca^{2+}$/CaM-mediated signalling in the presynaptic terminal, we next used KN93, an inhibitor that effectively blocks CaMKII (Marley and Thomson, 1996) (Fig. 5.2A). Although KN93 may have some activity against CaMKI and CaMKIV, there is no clear evidence for the function of these CaMKs in nerve terminals. Figures 5.2B and 5.2C show the different incubation protocols used to test for the effect of the drug. Data revealed that preincubation of KN93 is required for the drug to have an effect on ERK 1&2 activation. Whereas during a standard 10-minute incubation of synaptosomes with 5μM KN93, neither basal nor 4AP-induced ERK 1&2 activation was affected (Fig. 5.2B), preincubation with the drug resulted in significant reduction in both basal and 4AP-stimulated activation ERK 1&2 (Fig. 5.2C). In fact, results from KN93 preincubation showed that all of the basal ERK 1&2 activities that were dependent on $Ca^{2+}$ were inhibited by KN93 (basal, 100 ± 0%; basal/KN93, 70.0 ± 8.8%). This suggests that CaMKII is required in mediating the $Ca^{2+}$-dependent

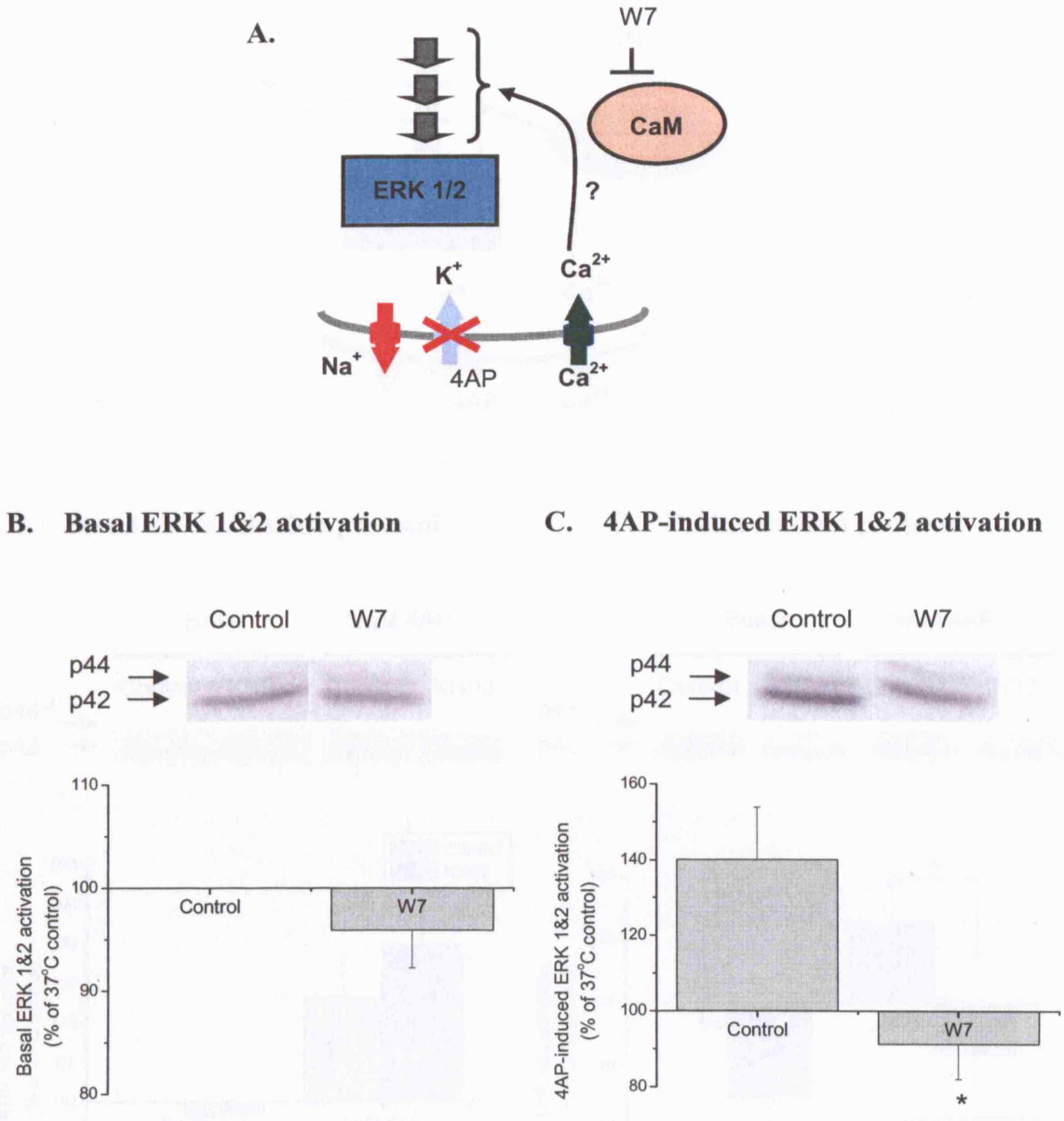



**Figure 5.1. Effect of W7, a CaM antagonist, on basal and 4AP-induced ERK 1&2 activation.**

**A.** Scheme showing proposed effects of W7 on ERK 1&2 activation. **B.** Phosphoimages and quantification for basal phospho-ERK with and without treatment of W7. **C.** Phosphoimages and quantification of 4AP-induced phospho-ERK with and without treatment of W7. Synaptosomes (0.3 mg/ml) were incubated in the absence (Control) and presence of 50μM W7 (W7), added at the start of the incubation. Depolarisation with 1mM 4AP was carried out at 10 min. Samples separation and immunoblotting with phospho-p44/42 MAPK (Thr202/Tyr204) antibody (NEB) was carried out as described in the Materials and Methods section. *$p<0.05$ compared to 37°C $CaCl_2$ control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with four independent synaptosomal preparations (n=4).

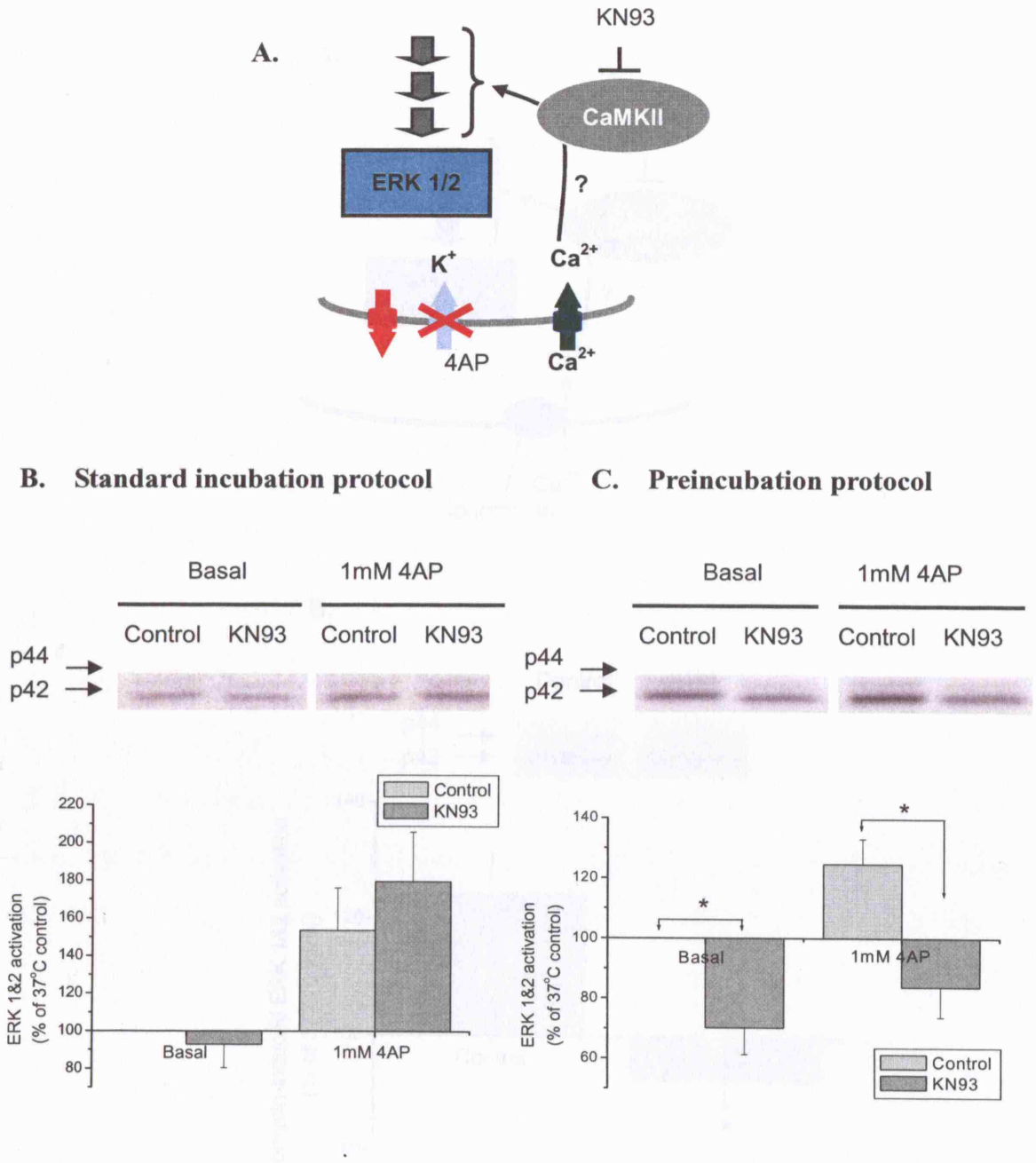



**Figure 5.2. Effect of CaMKII inhibitor KN93 on basal and 4AP-induced ERK 1&2 activation.**

**A.** Scheme showing proposed effects of KN93 on ERK 1&2 activation. **B.** Phosphoimages and quantification of basal and 4AP-induced phospho-ERK levels after standard incubation with KN93. **C.** Phosphoimages and quantification of basal and 4AP-induced phospho-ERK levels after preincubation with KN93. In the preincubation protocol, synaptosomes (0.3 mg/ml) were preincubated with 5μM KN93 before following the standard 10-minute incubation protocol as indicated in the Materials and Methods section. *$p<0.05$ compared to respective controls (unpaired Student t-test). Data were means ± SEM of experiments studied with three (n=3) and four (n=4) independent synaptosomal preparations for standard incubation and preincubation protocols respectively.

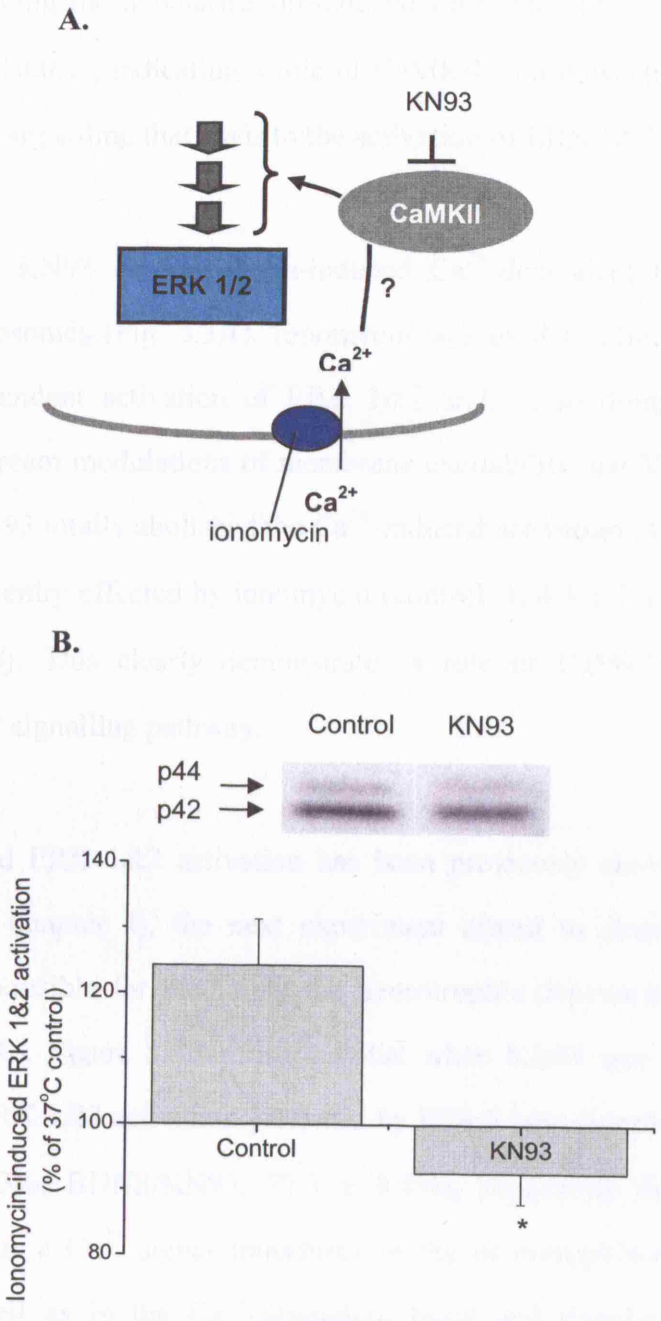



**Figure 5.3. Effect of KN93 on $Ca^{2+}$-dependent ERK 1&2 activation effected by ionomycin. A.** Scheme showing proposed effects of KN93 on ionomycin-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of ionomycin-induced phospho-ERK levels with and without KN93 treatment. Synaptosomes (0.3 mg/ml) were preincubated in the absence (Control) or presence of 5μM KN93 (KN93) before following the protocol for measuring $Ca^{2+}$-dependency with ionomycin (detailed in the Materials and Methods). *$p<0.05$ compared to 37°C ionomycin control (unpaired Student t-test). Data were means ± SEM of experiments studied with three independent synaptosomal preparations (n=3).

component of basal levels of ERK 1&2 activities. Similarly, KN93 was capable of significantly attenuating the depolarisation-induced ERK 1&2 (4AP, 124.6 ± 8.4%; 4AP/KN93, 83.8 ± 10.0%), indicating a role of CaMKII as a downstream $Ca^{2+}$/CaM effector for the $Ca^{2+}$ signalling that leads to the activation of ERK 1&2 (Fig. 5.2C).

Next, the effect of KN93 on ionomycin-induced $Ca^{2+}$-dependent ERK 1&2 was evaluated in synaptosomes (Fig. 5.3A). Ionomycin was used to effect a direct $Ca^{2+}$ influx for $Ca^{2+}$-dependent activation of ERK 1&2 and, in so doing, obviate any involvement of upstream modulations of membrane excitability and VGCCs. Results demonstrate that KN93 totally abolished the $Ca^{2+}$-induced activation of ERK 1&2 as a result of direct $Ca^{2+}$ entry effected by ionomycin (control, 124.3 ± 7.0%; KN93, 92.6 ± 4.6%) (Fig. 5.3B). This clearly demonstrates a role of CaMKII in the $Ca^{2+}$-dependent ERK 1&2 signalling pathway.

Since BDNF-induced ERK 1&2 activation has been previously shown to be $Ca^{2+}$-sensitive (shown in chapter 4), the next experiment aimed to determine whether CaMKII is also responsible for mediating the neurotrophin-dependent signalling to ERK 1&2 (Fig. 5.4A). Figure 5.4B illustrates that when KN93 was applied to the synaptosomes, the ERK 1&2 activation produced by BDNF was completely abolished (BDNF, 117.6 ± 5.3%; BDNF/KN93, 77.3 ± 8.4%), suggesting that CaMKII is important in acting as a $Ca^{2+}$ signal transducer in the neurotrophin-mediated ERK 1&2 pathway as well as in the $Ca^{2+}$-dependent basal and depolarisation-evoked pathways.

Having characterised CaM and CaMKII as the essential mediators in the $Ca^{2+}$-dependent ERK 1&2 pathway, we sought to identify whether or not the small GTP-binding protein Ras is also involved (Fig. 5.5A). To do this, synaptosomes were preincubated with 10μM lovastatin, a Ras inhibitor, before following the standard 10-

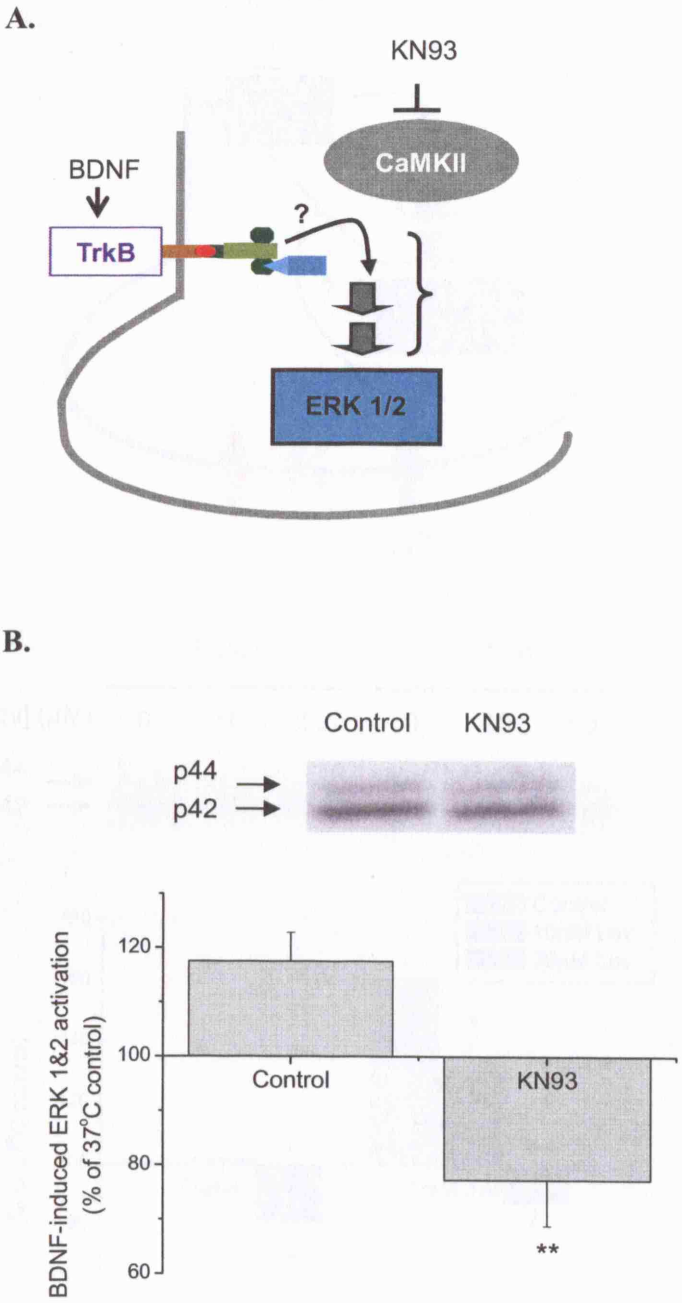



**Figure 5.4. Effect of KN93 on BDNF-induced ERK 1&2 activation.**

**A.** Scheme showing proposed effects of KN93 on BDNF-induced activation of ERK. **B.** Phosphoimages and quantification of BDNF-induced phospho-ERK levels with (KN93) and without (Control) treatment of KN93. Synaptosomes (0.3 mg/ml) were preincubated with 5μM KN93 before the standard 10-minute incubation protocol. **$p<0.01$ compared to 37°C BDNF control (unpaired Student t-test). Data were means ± SEM of experiments studied with five independent synaptosomal preparations(n=5).

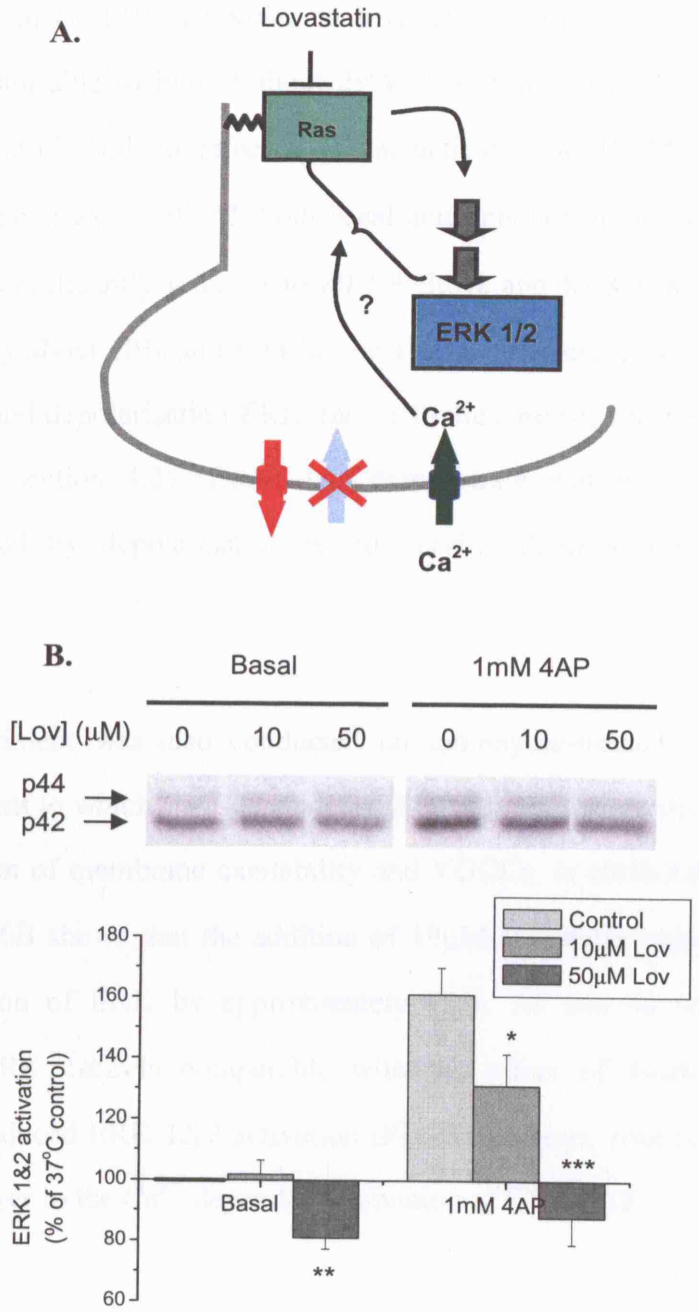



**Figure 5.5. Effect of increasing concentration of lovastatin, a Ras inhibitor, on basal and 4AP-induced ERK 1&2 activation. A.** Scheme showing proposed effects of lovastatin on ERK 1&2 activation. **B.** Phosphoimages and quantification of basal and 4AP-induced phospho-ERK levels with or without 10μM or 50μM lovastatin (Lov) addition. Synaptosomes (0.3 mg/ml) were preincubated with lovastatin (0, 10 or 50μM) before the standard incubation protocol. *p<0.05, **p<0.01, ***p<0.001 compared to respective controls (unpaired Student t-test). Data were means ± SEM of experiments studied with three independent synaptosomal preparations (n=3).

minute incubation protocol. Figure 5.5B shows the relative sensitivity of basal and depolarisation-induced ERK activities to lovastatin. Results revealed that the Ras inhibitor, although able to inhibit about 29% of 4AP-induced ERK 1&2 activation compared to control, had no effect on basal activation at 10μM. However, when lovastatin was increased to 50μM, both basal and depolarisation-induced ERK 1&2 activation was significantly reduced to 80.9 ± 3.6% and 87.8 ± 8.7% respectively. This reduction by about 20% and 12% below 37°C control corresponds with the level to which basal and depolarisation ERK 1&2 activities are seen to be $Ca^{2+}$-dependent (see chapter 4, section 4.3). These data demonstrate that basal and ERK 1&2 activation evoked by depolarisation is, to varying degrees, contingent on Ras activation.

A similar experiment was also conducted on ionomycin-treated synaptosomes to examine the extent to which $Ca^{2+}$-dependent ERK 1&2 activation, that is independent of the modulation of membrane excitability and VGCCs, is attributable to Ras (Fig. 5.6A). Figure 5.6B shows that the addition of 10μM lovastatin reduced ionomycin-induced activation of ERK by approximately 43%. As can be seen, this partial inhibition on ERK 1&2 is comparable with the effect of 10μM lovastatin on depolarisation-induced ERK 1&2 activation (Fig. 5.5B), thus, confirming that Ras is an important player in the $Ca^{2+}$-dependent activation of ERK 1&2.

Next, we determined the role of PI3K in mediating $Ca^{2+}$-dependent activation of ERK 1&2 using the PI3K inhibitor LY29004 (Sanchez-Margalet et al., 1994) (Fig. 5.7A). Data reveals that upon incubation with 100μM LY29004, ionomycin-induced ERK 1&2 activation is significantly attenuated compared to the control (control, 171.04 ± 19.36 %; LY29004, 85.69 ± 5.76 %) (Fig. 5.7B). This suggests that PI3K plays an important part in the regulation of $Ca^{2+}$ signalling to ERK 1&2 downstream of $Ca^{2+}$ entry.

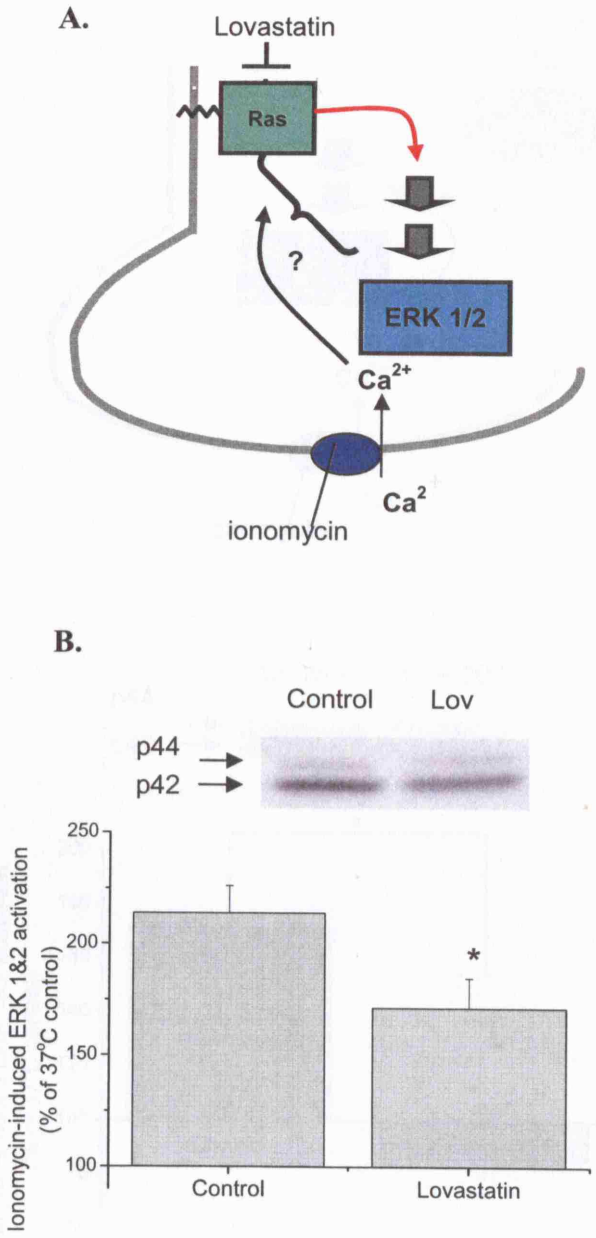



**Figure 5.6. Effect of Ras inhibitor, lovastatin, on activation of ERK 1&2 by direct $Ca^{2+}$ entry effected by ionophore ionomycin. A.** Scheme showing proposed effects of lovastatin on ionomycin-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of ionomycin-induced phospho-ERK levels in the absence and presence of lovastatin. Synaptosomes (0.3 mg/ml) were preincubated with (Lovastatin) or without (Control) 10μM lovastatin before following the protocol for measuring $Ca^{2+}$-dependency of ERK 1&2, as indicated in the Materials and Methods. *$p<0.05$ compared to 37°C ionomycin control (unpaired Student t-test). Data were means ± SEM of experiments studied with seven (n=7) and six (n=6) independent synaptosomal preparations for control and lovastatin respectively.

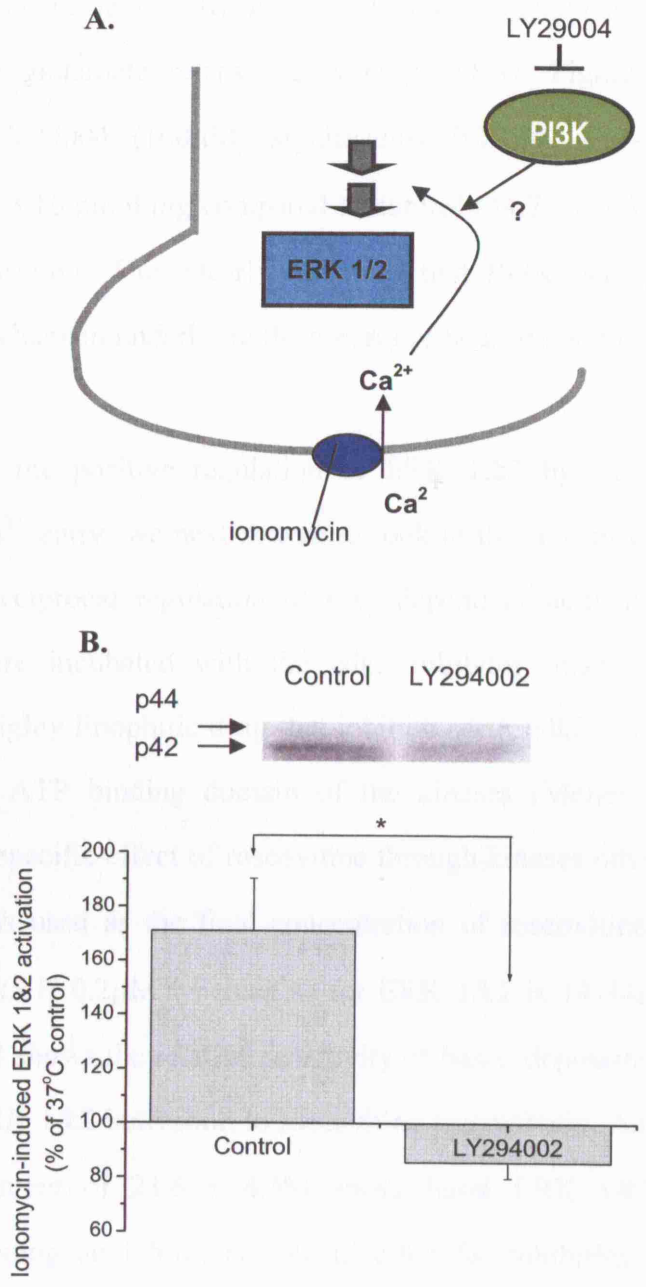



**Figure 5.7. Effect of PI3K inhibitor, LY29004, on activation of ERK 1&2 by direct $Ca^{2+}$ entry effected by ionophore ionomycin. A.** Scheme showing proposed effects of LY29004 on ionomycin-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of ionomycin-induced phospho-ERK levels with or without incubation of LY29004. Synaptosomes (0.3 mg/ml) were incubated in the presence (LY29004) or absence (Control) of 100μM LY29004 before following the protocol for measuring $Ca^{2+}$-dependency of ERK 1&2, as described in the Materials and Methods. *$p<0.05$ compared to 37°C ionomycin control (unpaired Student t-test). Data were means ± SEM of three (n=3) independent synaptosomal preparations.

We also confirmed the role of PI3K in the modulation of presynaptic neurotransmitter release using the glutamate release assay (Fig. 5.8A). Figure 5.8B shows that treatment with LY29004 (100μM) significantly inhibits 4AP-evoked glutamate release to 14.88 ± 3.16 nmol/mg compared to control (34.74 ± 4.52 nmol/mg) at 240 sec after depolarisation. This clearly indicates that PI3K acts presynaptically to function in the mechanism underlying the control of neurotransmitter release.

Having identified the positive regulation of ERK 1&2 by CaM, Ras and PI3K downstream of $Ca^{2+}$ entry, we next sought to look at the role of cdk5 as a potential mediator in the reciprocal regulation of $Ca^{2+}$-dependent activation of ERK 1&2. Synaptosomes were incubated with the cdk5 inhibitor, roscovitine (Fig. 5.9A). Roscovitine is a highly lipophilic drug that inhibits cdc2, cdk2/5 and ERK 1 & 2 by competing at the ATP binding domain of the kinases (Meijer et al., 1997). To minimise the non-specific effect of roscovitine through kinases other than cdk5, (Yan Z 2002), 5μM was used as the final concentration of roscovitine given that $K_i$ of roscovitine for cdk5 is 0.2μM whereas $K_i$ for ERK 1&2 is 14-34μM (Meijer et al., 1997). Figure 5.9B shows the relative sensitivity of basal, depolarisation-induced and BDNF-induced ERK 1&2 activation to roscovitine respectively. As can be seen, data show an enhancement of 23.6 ± 4.3% above basal ERK 1&2 activation with roscovitine, suggesting an inhibitory role of cdk5 for inhibiting ERK 1&2 under resting conditions. However, the role of cdk5 appears to be different in stimulated conditions when ERK 1&2 activation was induced by depolarisation or by BDNF. Results show that depolarisation-induced ERK 1&2 activation was reduced by about 13% after treatment with roscovitine and this was found to be statistically significant. Roscovitine was also found to inhibit BDNF-mediated ERK 1&2 activation, although this was statistically non-significant (BDNF, 112.7 ± 4.3%; BDNF/Ros, 88.6 ± 8.8%). These data suggest that cdk5 may either play a facilitatory role in the regulation of

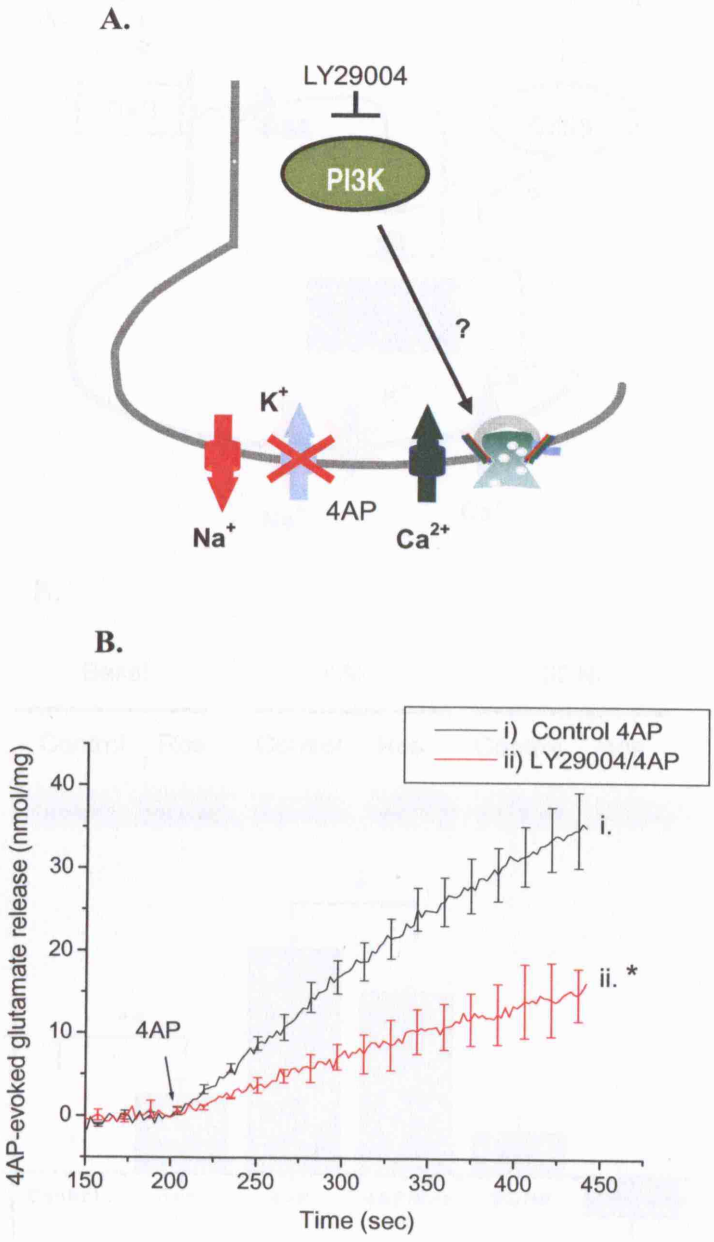



**Figure 5.8. Effect of LY29004 on 4AP-evoked glutamate release.**

**A.** Scheme showing proposing effect of PI3K inhibitor LY29004 on depolarisation-induced glutamate release. **B.** Glutamate release: i) Control 4AP; ii) LY29004/4AP. Synaptosomes (0.1 mg/ml) were incubated in the presence of 1mM of $CaCl_2$ and release was followed by on-line fluorimetry under standard conditions as indicated in the Materials and Methods section. Glutamate release was elicited with the addition of 1mM 4AP in the absence (i) or presence (ii) of 100μM LY29004 added at the start of the incubation. *$p<0.05$ compared to 4AP control (unpaired Student t-test). Data were means ± SEM of experiments studied with three independent synaptosomal preparations (n=3).

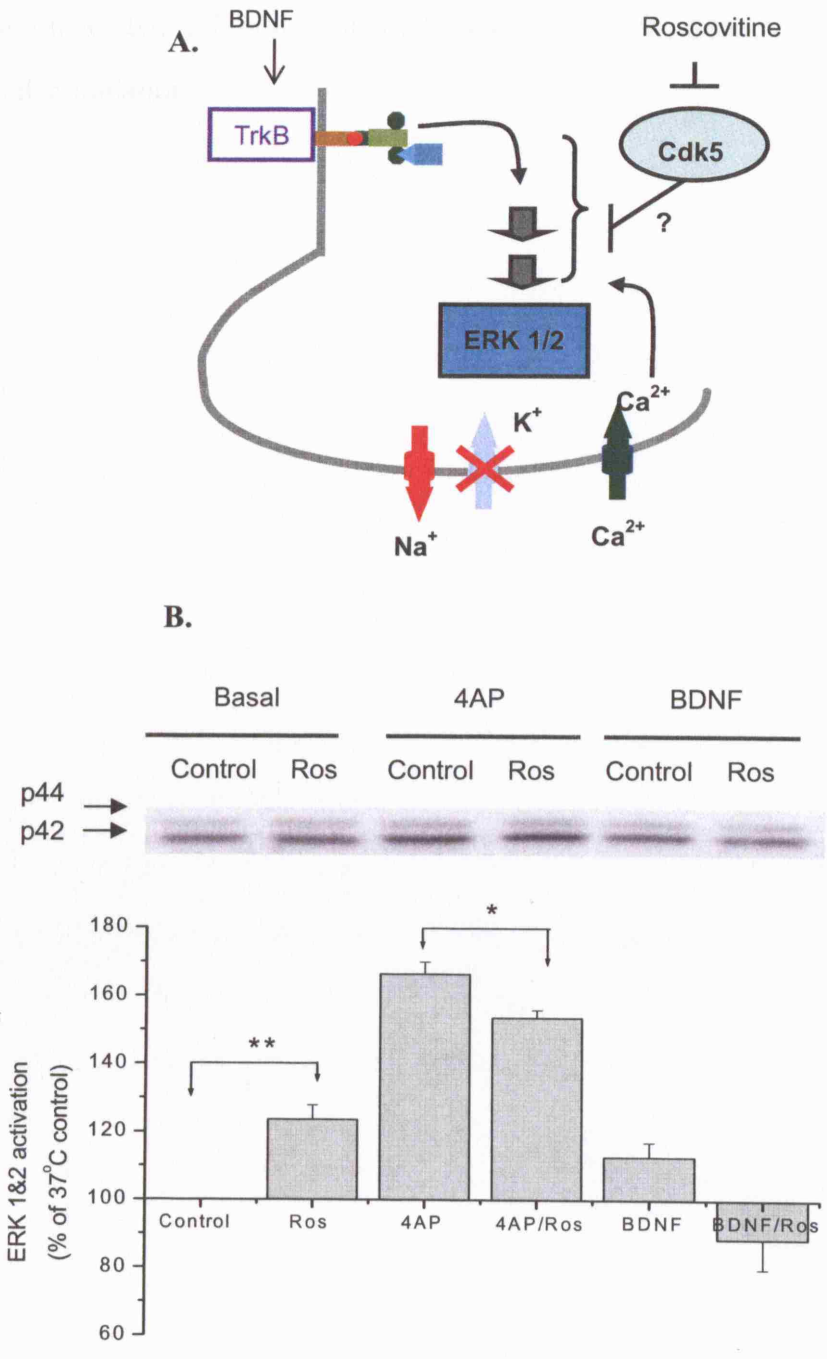



**Figure 5.9. Effect of roscovitine, a cdk5 inhibitor, on basal, 4AP-induced and BDNF-mediated ERK 1&2 activation. A.** Scheme showing proposed effects of roscovitine on basal, 4AP-induced and BDNF-induced activation of ERK 1&2. **B.** Phosphoimages and quantification of phospho-ERK following with and without treatment of roscovitine. Synaptosomes (0.3 mg/ml) were incubated with 1% DMSO (Control) or 5μM roscovitine (Ros) in the absence and presence of 200ng/ml BDNF. 4AP (1mM) was added at 10 min to depolarise the synaptosomes. *$p<0.05$ compared to 4AP control, **$p<0.01$ compared to 37°C control (unpaired Student t-test). Data were means ± SEM of experiments studied with three independent synaptosomal preparations (n=3).

depolarisation and BDNF-induced ERK 1&2 or that stimulation somehow overcomes the cdk5-mediated inhibition of ERK 1&2 following depolarisation or BDNF-mediated stimulation.

## 5.4. Discussion

The aim of this chapter was to identify the intermediary elements by which $Ca^{2+}$ signals can be transduced to initiate the activation of ERK 1&2 in the presynaptic nerve terminals. Our results have implied a possible role of calmodulin (CaM) in both basal and 4AP-induced ERK 1&2 activation using the CaM antagonist, W7. We show that the addition of W7 totally inhibited depolarisation-induced activation of ERK 1&2 and that there was a tendency for the inhibitor to reduce basal ERK 1&2 activities (albeit statistically non-significantly). Although previously a number of groups have reported the non-specific effect of W7 on functionally blocking VGCCs to reduce $Ca^{2+}$ current induced by depolarisation (Greenberg et al., 1987), data shown by Quinn *et al.* suggested that the effect of W7 was specific to CaM as they showed that ionomycin or $Ca^{2+}$-induced ERK activation was sensitive to W7 (Quinn et al., 2002). This function of CaM in ERK 1&2 signalling downstream of $Ca^{2+}$ entry and receptor activation at the plasma membrane was also supported by reports demonstrating that CaM modulated ERK 1&2 activation following membrane depolarisation in PC12 cells independently of Trk receptors activation (Egea et al., 2000; Egea et al., 1999). Taking these studies into consideration, our results appear to be consistent with the hypothesis that CaM is a mediator of the $Ca^{2+}$-dependent signalling in the ERK 1&2 activation pathway. This may, in turn, underlie a mechanism by which neurotransmitter release is regulated, as suggested by previous evidence for the involvement of CaM in neurotransmitter release (Sihra and Pearson, 1995; Llinas et al., 1991; Lin et al., 1990; Nichols et al., 1990).

In the present study, we have shown the involvement of CaMKII, one of the major downstream CaM effectors, in the $Ca^{2+}$-dependent stimulation of the ERK cascade. Data demonstrated that both basal and depolarisation-induced ERK 1&2 activation were attenuated when synaptosomes were preincubated with KN93, a CaMKII inhibitor. The fact that the entire $Ca^{2+}$-dependent component of basal activities (20%

of basal ERK 1&2 activation, as shown in chapter 4) was inhibited and that all of the activation of ERK 1&2 raised by depolarisation were blocked by KN93, indicates the importance of CaMKII in mediating ERK 1&2 signalling. This is in agreement with studies in vascular smooth muscle, which have implicated CaMKII as a major player in the $Ca^{2+}$-dependent ERK 1&2 activation, also using KN93 (Ginnan and Singer, 2002). Furthermore, our results show that KN93 completely abolishes the $Ca^{2+}$-induced activation of ERK 1&2 as a result of direct $Ca^{2+}$ entry effected by ionomycin. This suggests that CaMKII is responsible in mediating the direct $Ca^{2+}$-dependent signalling to ERK 1&2 downstream of the plasma membrane. Thus, together with the observation that CaM may be involved in the $Ca^{2+}$-dependent ERK cascade, these data provide corroboration for the suggestion that one of the signalling mechanisms to ERK 1&2 in response to $Ca^{2+}$ signals is operating through CaM and CaMKII. This model is further supported by our findings that the $Ca^{2+}$-sensitive BDNF-mediated ERK 1&2 activation was also contingent on CaMKII activities given that KN93 was capable of attenuating the activation of ERK 1&2 following neurotrophin stimulation. The sensitivity of the BDNF-induced activation of ERK to KN93 seems to be consistent with the previously suggested role of $Ca^{2+}$ as a permissive input, allowing more effective BDNF-mediated signalling to occur through the activation of CaMKII (see chapter 4).

So far, the present work has identified both CaM and CaMKII as potential mediators in the $Ca^{2+}$-dependent ERK 1&2 signalling. But where in the ERK pathway do these mediators impinge to transduce signals necessary for the subsequent ERK 1&2 activation? One possibility is Ras, the upstream regulator of ERK 1&2 proposed in chapter 3. Previously, $Ca^{2+}$/CaM signals have been shown to control the activity of Ras through activation of upstream Ras regulators such as Ras-GRF or Ras-GAP (Bos, 1998; Chen et al., 1998; Farnsworth et al., 1995). Furthermore, SynGAP has been demonstrated to be phosphorylated, and thus, inactivated by CaMKII, leading to an inhibition of ERK 1&2 activation (Oh et al., 2004; Bos, 1998; Chen et al., 1998).

Having identified the involvement of Ras in BDNF-stimulated ERK 1&2 activation earlier in the thesis (see chapter 3), the results in this chapter indicate that the activation of Ras was also necessary for both basal and depolarisation-induced ERK 1&2 signalling although the degree to which effects were attributable to Ras were variable. Furthermore, ionomycin-induced $Ca^{2+}$-dependent ERK activation was inhibited by the addition of lovastatin, the Ras inhibitor. These results suggest that Ras plays an important role in mediating the $Ca^{2+}$-dependent signalling to ERK 1&2 and may represent a point at which different cascades, including the basal, depolarisation-induced and neurotrophin-mediated pathway, converge to mediate downstream ERK 1&2 activation.

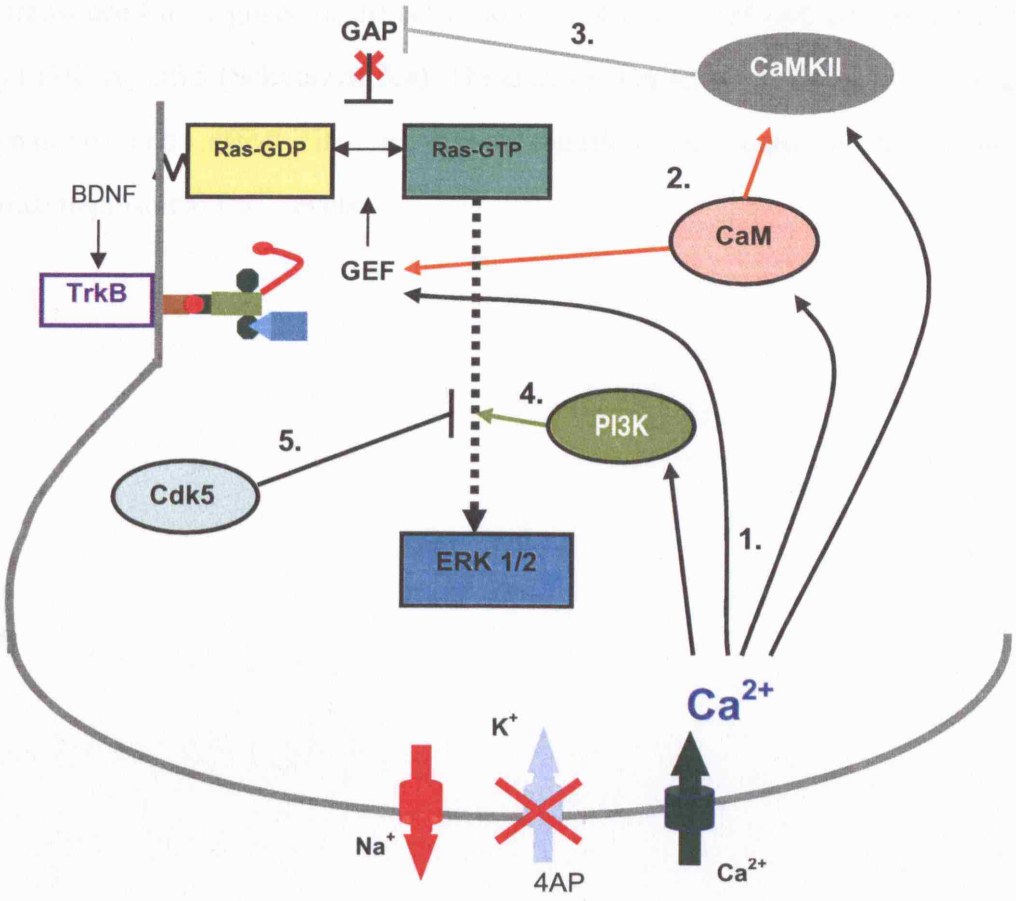
Although data from the CaMKII experiments showed that the CaMKII inhibitor seems to block the majority of the $Ca^{2+}$-component of basal, depolarisation and ionomycin-induced ERK 1&2 activation, this may not completely rule out phosphatidyl-inositol-3-kinase (PI3K) and/or protein kinase C (PKC) as other potential mediators that may be involved in the $Ca^{2+}$-dependent activation of ERK. Indeed, our results demonstrated that addition of LY29004, a PI3K inhibitor, significantly attenuated ionomycin-induced activation of ERK 1&2, indicating that PI3K is involved in the modulation of $Ca^{2+}$-dependent ERK 1&2 activation. Presynaptically, we showed that inhibition of PI3K with LY29004 also led to a reduction in depolarisation-induced glutamate release from cerebrocortical synaptosomes. This appears to be consistent with findings that have demonstrated the importance of PI3K in controlling the SV delivery to the RRP from the RP (Cousin et al., 2003). Thus, these data indicate a role of PI3K in the control of neurotransmitter release, such that PI3K may serve as a mediator for $Ca^{2+}$-dependent ERK 1&2 signalling, and thereby, affecting synapsin-mediated regulation of SV exocytosis downstream.

The modulatory role of PI3K in the regulation of ERK 1&2 signalling has been

previously evident in Cos-7 cells where overexpression of PI3K has been shown to constitutively activate the ERK cascade, whereas a catalytically inactive PI3K mutant blocks GPCR-mediated ERK activation (Lopez-Ilasaca et al., 1997). Furthermore, NMDA or hydrogen peroxide-mediated signalling to ERK 1&2 has been shown to require the presence of $Ca^{2+}$ and PI3K in cultured striatal neurons (Crossthwaite et al., 2002; Perkinton et al., 2002; Chandler et al., 2001). Thus, it is likely that the PI3K signalling pathway may crosstalk with the ERK 1&2 cascade in the nerve terminal to regulate presynaptic functions. One possible mechanism by which PI3K may mediate ERK 1&2 signalling is by its ability to regulate activities of upstream regulators of ERK such as Raf kinases or MEK (Bondeva et al., 1998; Hu et al., 1995). In addition, studies have shown that PI3K may activate its downstream target Akt to cause phosphorylation of transcription factors which may then upregulate proteins involved in ERK 1&2 activation (Perkinton et al., 2002; Bonni et al., 1999). Further work, however, is required to examine the exact mechanisms of how PI3K is involved in mediating $Ca^{2+}$-dependent activation of ERK 1&2.

Apart from mediation through Ras and PI3K, $Ca^{2+}$-dependent signalling to ERK 1&2 may also occur through the activation of PKC. In this regard, studies have shown PKC to have a modulatory role on ERK signalling where PKC positively regulates the activation of ERK 1&2 in a number of systems (Brodie et al., 1999; Roberson et al., 1999). In cerebrocortical synaptosomes, this PKC-mediated ERK 1&2 signalling has been demonstrated and is posited to contribute to the mechanism underlying the regulation of presynaptic neurotransmitter release (Davies, 2004). However, the level at which PKC may act to facilitate ERK 1&2 activation is yet to be established. If modulation of ERK 1&2 signalling by PKC occurs at the level of Raf-1, PKC-mediated activation of ERK should not be lovastatin-sensitive. Further experiments are warranted to address this issue directly.

In contrast to the positive regulation of $Ca^{2+}$-dependent activation of ERK 1&2 by CaMKII, Ras and PI3K, our data also suggest a way by which ERK 1&2 can be reciprocally regulated by cdk5. Using roscovitine, a cdk5 inhibitor, we show that cdk5 was involved in negatively regulating ERK 1&2 signalling during basal conditions, as suggested by the enhancement of basal ERK 1&2 activities after synaptosomal treatment with the drug. This is consistent with reports showing that cdk5 can downregulate ERK 1&2 activities both *in vitro* and *in vivo* (using p35 knockout mice) by mediating phosphorylation of MEK 1&2 (Sharma et al., 2002) and/or phosphorylation of Ras-GRF (Kesavapany et al., 2004) following its activation by $Ca^{2+}$-dependent proteolysis of its activator p35. However, the effect of roscovitine was different on depolarisation-induced ERK 1&2. This was evinced by the ability of roscovitine to significantly attenuate ERK 1&2 activation evoked by depolarisation. Similarly, following synaptosomal stimulation by BDNF, data showed a tendency for roscovitine to inhibit the activation of ERK 1&2, although this was not found to be statistically significant. These data suggest that cdk5 may play a different role during basal and stimulated conditions where it exerts reciprocal control over ERK 1&2 at "resting" state, but during stimulated conditions, the role of cdk5 on ERK 1&2 reverses to facilitate rather than to inhibit ERK 1&2 signalling. Alternatively, stimulation by depolarisation or neurotrophin addition may overcome the inhibitory effects of cdk5 on ERK 1&2 through an unknown mechanism. However, given that there is evidence that roscovitine directly initiates $Ca^{2+}$ influx through P/Q type VGCCs independent of cdk5 action (Yan et al., 2002; Tomizawa et al., 2002), questions arise as to the relative specificity of the cdk5 inhibitor. More work will, therefore, be required to confirm the physiological relevance of cdk5 on ERK 1&2 cross-signalling. Experiments using ionomycin to by-pass upstream events at the plasma membrane will help to explain the implication of cdk5 in mediating signals for the pathway.




**Schematic 5.4. The mediators in $Ca^{2+}$-dependent activation of ERK 1&2.** 1.) $Ca^{2+}$ signal can activate downstream mediators such as CaMKII in conjunction with CaM, PI3K as well as binds directly to Ras-GRF to activate Ras-ERK signalling; 2.) $Ca^{2+}$/CaM may act by binding to and activate CaMKII and Ras-GEF; 3.) CaMKII may act by phosphorylating and inhibiting SynGAP, a GAP for Ras, and subsequently, increasing Ras-ERK signalling; 4.) PI3K is a $Ca^{2+}$ effector that acts to increase ERK activation, although the site of its action is yet to be determined; 5.) cdk5 acts as a negative mediator for ERK 1&2 activation by possibly dephosphorylating, and thus, inhibiting MEK 1&2 following its $Ca^{2+}$-dependent activation.

This chapter had explored various mediators by which the presynaptic terminal uses to transduce $Ca^{2+}$ signals for the activation of ERK 1&2: i) CaM; ii) CaMKII; iii) Ras; iv) PI3K; iv) cdk5 **(Schematic 5.4)**. These mediators all act together to fine-tune ERK signalling and affect its activation duration in response to changes in intrasynaptosomal $Ca^{2+}$ levels.

# Chapter 6

# INVOLVEMENT OF NON-RECEPTOR TYROSINE KINASES IN ERK 1&2 SIGNALLING

## 6.1. Introduction

ERK 1&2 signalling pathway has been established as a multi-step system involving layers of kinase regulation. Activation of ERK 1&2 can be mediated through dual phosphorylation of specific tyrosine and threonine residues in its signature sequence by upstream MEKs. ERK 1&2 signalling pathway can be activated in response to many stimuli including the activation of receptor tyrosine kinases (RTKs) (Huang and Reichardt, 2003), G-protein coupled receptors (GPCRs) (Della Rocca et al., 1999; Dikic et al., 1996), glutamate receptors (Perkinton et al., 2002; Chandler et al., 2001; Vanhoutte et al., 1999; English and Sweatt, 1996), as well as $Ca^{2+}$ influx as a result of membrane depolarisation ((Baldassa et al., 2003; Egea et al., 1999), see chapter 4). But, how do all these diverse upstream signals lead to a common activation of ERK 1&2 by dual tyrosine/threonine phosphorylation? In several systems, a role for the non-receptor tyrosine kinases (nRTKs) has been proposed as an essential intermediary layer of signalling involved in ERK activation. In particular, studies point to the importance of nRTKs of the Src family (SFK) in the upstream mediation of signalling to ERK 1&2 (Crossthwaite et al., 2004; Perkinton et al., 1999; Dikic et al., 1996; Finkbeiner and Greenberg, 1996; Lev et al., 1995).

SFKs have been shown to be widely expressed in CNS (Boxall and Lancaster, 1998). Presynaptically, they are found enriched in SVs and account for the majority of SV tyrosine kinase activity (Linstedt et al., 1992). In non-neuronal preparations, these nRTKs have been implicated in GPCR-induced signalling (Luttrell et al., 1996; Dikic et al., 1996). SFKs have also been found involved in NMDA-mediated signalling where they are rapidly activated following glutamate receptor activation in striatal neurons (Crossthwaite et al., 2004). Once activated, the nRTKs may provide a structural scaffold for the assembly of signalling complexes for downstream activation of ERKs (Luttrell et al., 1999; Crossthwaite et al., 2004). Studies in Cos-7 cells revealed that the activation of Ras-Raf-ERK signalling pathway can be initiated

following the Src-dependent tyrosine phosphorylation of Shc and the subsequent recruitment of Grb2 and Sos as a result of GPCR activation (Luttrell et al., 1996). Similarly, in vascular smooth muscle, the $Ca^{2+}$-dependent nRTK, Pyk2, has been suggested to be involved in the regulation of ERK 1&2 activation where angiotensin II promotes the formation of a complex between Pyk2 and the ras/ERK regulators Shc and Grb2 (Rocic et al., 2001). In addition, SFKs have been shown to relay signals to trigger ERK 1&2 cascade by a direct association between SFKs and modules in the ERK 1&2 pathway. This has become apparent with the ability of another member of SFK, Fyn to associate, with TrkB where the SH2 domain of the nRTK interacts with the intracellular domain of the RTK (Iwasaki et al., 1998). This association is dependent upon stimulation with BDNF and thus, suggests that Fyn is a downstream effector of TrkB activation. Furthermore, Src can interact with H-Ras in both brain extracts and *in vitro*, although interestingly, H-Ras was proposed in these studies to negatively regulate the activity of Src (Thornton et al., 2003).

Apart from acting in the receptor-mediated signalling, SFKs have also been suggested to transduce $Ca^{2+}$ signals to downstream activation of ERK. Studies have shown that SFKs are themselves activated in response to stimuli that causes the elevation of $Ca^{2+}$ (Siciliano et al., 1996; Dikic et al., 1996; Lev et al., 1995). Upon activation, SFKs have been demonstrated to act at two possible loci: i) at the level of $Ca^{2+}$ influx; ii) downstream of $Ca^{2+}$ influx whereby SFKs function to transduce $Ca^{2+}$ signals for the subsequent ERK 1&2 activation. Previous studies have shown that SFKs can regulate excitability and/or $Ca^{2+}$ influx directly, and thereby alter $Ca^{2+}$-dependent signaling cascade including those effecting ERK activation. This is evinced by the ability of SFKs to regulate the function and activities of voltage-gated ion channels such as $Ca^{2+}$ channels (Evans and Pocock, 1999; Cataldi et al., 1996) and potassium channels (Fadool et al., 1997). Alternatively, studies have shown that Pyk2 plays a role in depolarisation-induced activation of ERK where activation can be prevented in the Pyk2-defective PC12 cells (Barsacchi et al., 1999). This is in agreement with studies

illustrating that $Ca^{2+}$ stimulation of ERK 1&2 occurring through AMPA receptor activation and VGCCs is dependent on SFKs (Perkinton et al., 1999).

Together, the evidence indicates an important role of SFKs in the regulation of neural excitability and synaptic plasticity, either by acting as a hub to scaffold elements and mediate signalling, or to physically associate with and induce tyrosine phosphorylation of effectors or signalling molecules to establish crosstalk. This chapter investigates the role of SFKs in the mechanisms underlying presynaptic ERK signalling and the control of glutamate release. We sought to determine whether SFKs have a function in the regulation of presynaptic ERK 1&2 signalling in synaptosomes. In addition, we examined the role of SFKs in mediating the $Ca^{2+}$-induced ERK signalling which has been observed in the foregoing chapters, in depolarisation and ionomycin-induced conditions, as well as during basal conditions. Finally, we evaluated whether there is any inter-dependence and/or cross-talk between the TrkB-mediated and the SFKs-mediated ERK activation.

## 6.2. Materials and Methods

### 6.2.1. Synaptosomal preparation

Synaptosomes were prepared as described in section 2.1.1.

### 6.2.2. Glutamate release experiment

Glutamate release was assayed as described in section 2.2.1. Synaptosomes were resuspended in HBM containing 1mg/ml BSA and incubated in the presence of 50U/ml GDH and 2mM NADP. BDNF (200ng/ml) or PP2 (10μM, preincubation required) was added at the start of the incubation. At 3 min, 1mM $CaCl_2$ was added and depolarisation was achieved with addition of 1mM 4AP at 10 min.

### 6.2.3. Standard incubation for ERK 1&2 activation/phosphorylation

The standard incubation protocol was followed as indicated in section 2.4.1. Synaptosomes were resuspended in HBM containing 1mg/ml BSA and incubated at 37°C with BDNF (200ng/ml), PP2 (10μM) or PP3 (10μM) added at the start of the incubation. $CaCl_2$ (1mM) was then added at 3 min before secretagogue 4AP was added at 10 min. Reactions were terminated with the addition of sample buffer 1 min after the secretagogue.

### 6.2.4. Protocol measuring $Ca^{2+}$-dependency of ERK 1&2 with ionomycin and $Ca^{2+}$-buffers

Synaptosomes were incubated in the HBM buffer in the presence of 1mg/ml BSA for 10 minutes as described in section 2.4.3. EGTA (50μM) was added 3 min after which 5μM ionomycin was added at 4 min. Calcium (500μM final free $Ca^{2+}$ concentration) was added at 10 min and reaction was stopped with the addition of sample buffer. Drugs of interest were added at the time shown in the figure legend.

### 6.2.5. Preincubation protocol

Preincubation of synaptosomes were carried out at 37°C in HBM containing 1mg/ml BSA in the presence of 1mM $CaCl_2$. DMSO (1%), PP2 (10μM) or PP3 (10μM) was added at 10 minutes after which synaptosomes were further incubated for 20 minutes. Synaptosomes were then pelleted at 10,000xg. Supernatants were discarded and the pellets cooled back to 4°C for 5 min before continuing with the standard incubation protocol.

### 6.2.6. SDS-PAGE and Immunoblotting

Proteins were separated on SDS-PAGE and subjected to immunoblotting using phospho-specific ERK 1&2 antibody as described in detail in sections 2.4.5 and 2.4.6.

### 6.2.7. Statistical analysis

Statistical analysis was carried out with Student's paired t-tests.

### 6.2.8. Reagents

A 1mg/ml stock solution of BDNF was made in buffer containing PBS before diluting to a working solution of 20μg/ml in water. BDNF was used at final concentration of 200ng/ml.

A stock of 5mM ionomycin was made in DMSO and a working solution of 500μM was obtained using HBM. Ionomycin was used at 5μM as a final concentration.

A 10mM stock solution of PP2 was obtained in DMSO after which a working solution of 1mM was made using HBM. PP2 was used at 10μM final concentration.

A 10mM stock solution of PP3 was made using DMSO and diluted further to 1mM as a working solution using HBM. PP3 was used at final concentration of 10μM.

## 6.3. Results

To study whether or not nRTK kinase activity stimulates the activities of presynaptic ERK 1&2, general antagonists of SFKs were used. In order to establish the experimental conditions required, we first examined the primary readout of presynaptic function, namely, neurotransmitter release from the cerebrocortical synaptosomes. Glutamate release was monitored using on-line fluorimetry with 10μM, 20μM and 100μM of PP2, a Src family nRTK inhibitor (Hanke et al., 1996) (Fig. 6.1A). Figures 6.1B and 6.1C show the relative sensitivity of basal glutamate release to PP2. Thus, at 10μM and 20μM, PP2 alone increased glutamate release to 2.8 ± 0.3 nmol/mg and 5.2 ± 1.1 nmol/mg glutamate per synaptosomal protein respectively after 200sec compared to the control (2.1 ± 1.2 nmol/mg). At 100μM PP2, the release of glutamate was increased to 24.3 ± 2.1 nmol/mg. To confirm the effect of PP2 on basal glutamate release, the inactive analogue of PP2, PP3 (Traxler et al., 1997), was used as a control. Figure 6.1D shows that application of 10μM PP2 had little effect on basal glutamate release (control, 0.04 ± 0.3 nmol/mg; PP2, 4.5 ± 1.0 nmol/mg), comparable with the effect produced by the inactive analogue PP3 (4.6 ± 1.0 nmol/mg). Taken together, the observation suggests that the antagonist PP2 appears to affect glutamate release in a dose-dependent manner. Whereas at low concentrations of PP2, the drug has no effect on basal glutamate release, PP2 is more likely to be non-specific at higher concentrations. At 10μM, PP2 and PP3 have very modest and similar effects on basal levels of glutamate release. Although the basis for this minor non-specific action of either inhibitor has not been elucidated, PP2 was used at 10μM to analyse the function of SFKs in subsequent studies.

Accordingly, next, the role of SFKs in depolarisation-induced glutamate release was evaluated using PP2 and PP3 (Fig. 6.2A). Data revealed a control 4AP release of 26.4 ± 0.8 nmol/mg at 240sec after depolarisation. Increasing concentrations of PP2

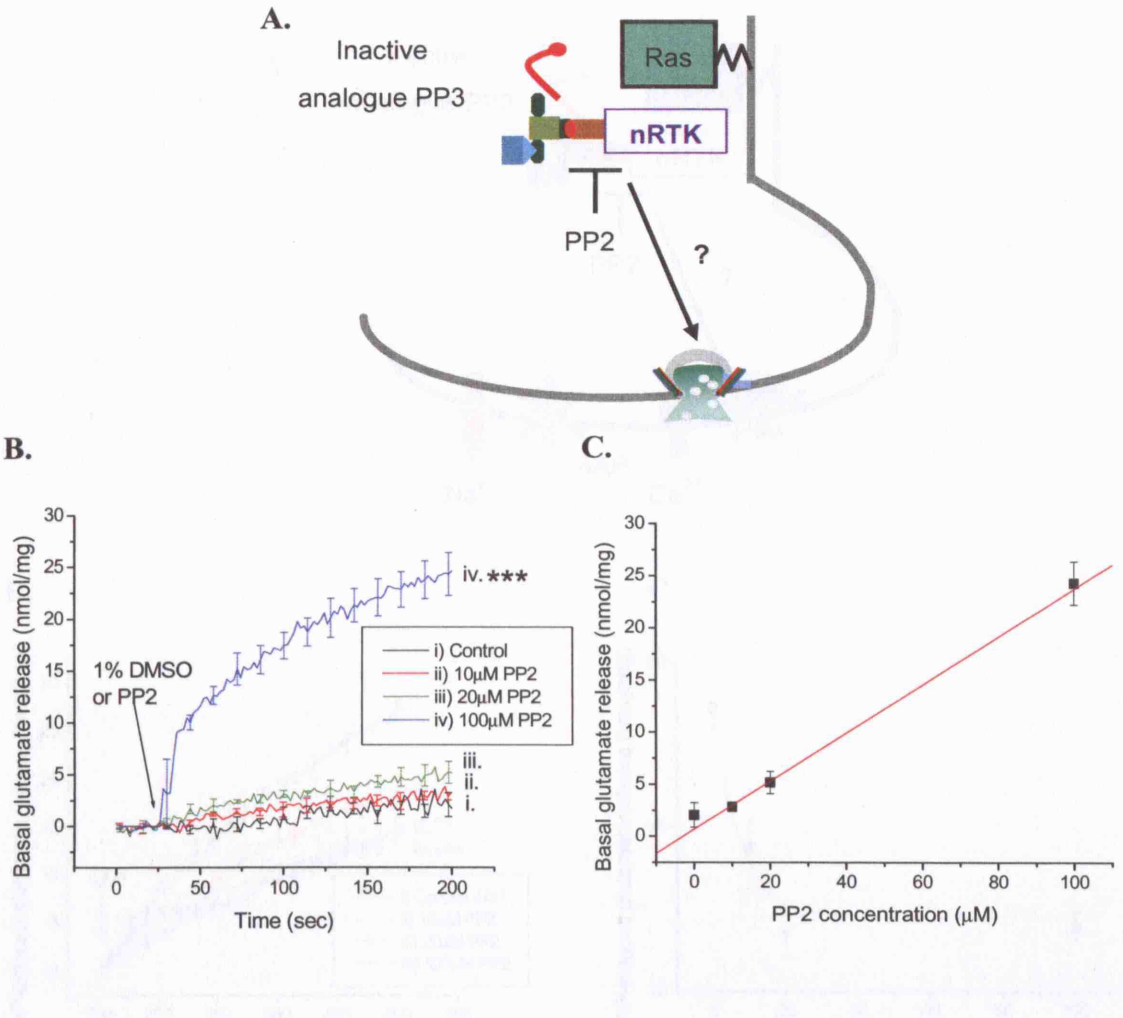
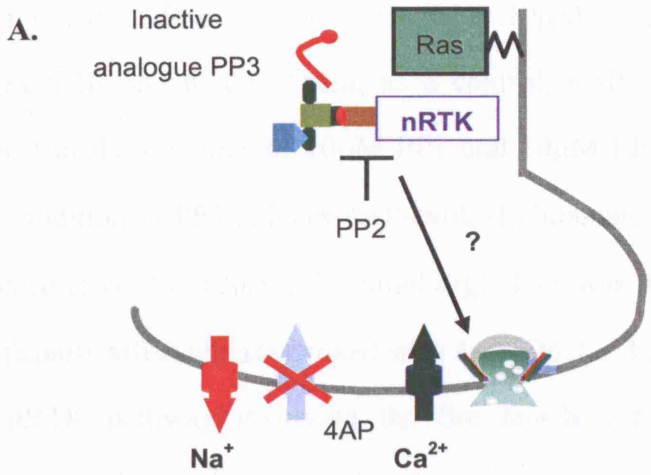



**Figure 6.1. Dose dependency of Src family kinase inhibitor PP2 and inactive analogue PP3 on basal glutamate release.** **A.** Scheme showing proposed effects of PP2 and PP3 on basal glutamate release. **B.** Glutamate release: i) Control; ii) 10μM PP2; iii) 20μM PP2; iv) 100μM PP2. Synaptosomes (0.15 mg/ml) were incubated in the presence of 1mM of $CaCl_2$ and release was followed by on-line fluorimetry as indicated in the Materials and Methods section. Basal glutamate release was monitored in the absence (Control) or presence (PP2) of PP2. **C.** Dose dependency of Src family kinase inhibitor PP2 at 200sec. Curve fit, linear fit. **D.** Glutamate release: i) control; ii) 10μM PP2; iii) 10μM PP3. ***$p<0.001$ compared to 37°C control at 200 sec (unpaired Student t-test). Data represent means ± SEM of experiments carried out with three independent synaptosomal preparations (n=3).

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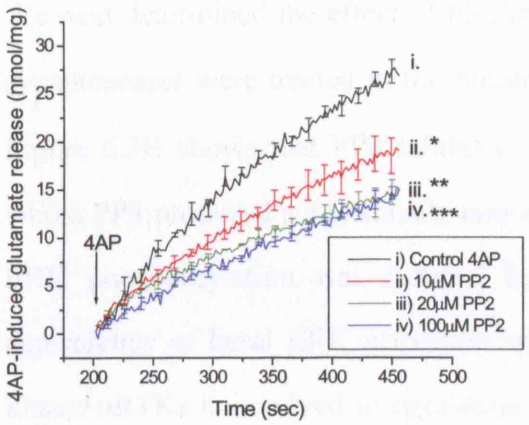


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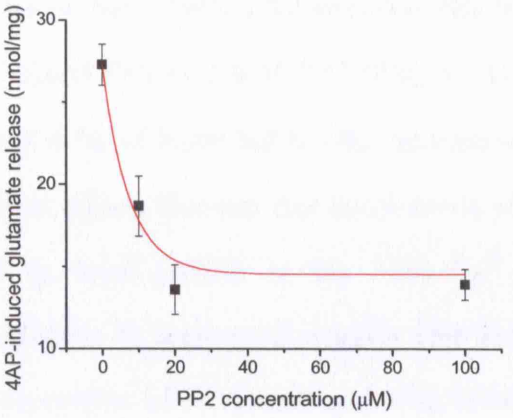


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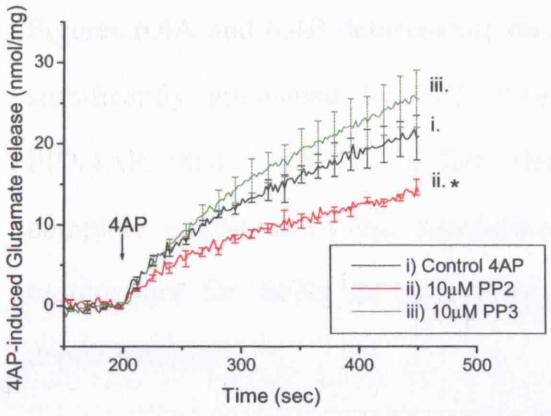



**Figure 6.2. Dose dependency of Src family kinase inhibitor PP2 and inactive analogue PP3 on 4AP-induced glutamate release. A.** Scheme showing proposed effect of PP2 and PP3 on depolarisation-induced glutamate release. **B.** Glutamate release: i) Control 4AP; ii) 10μM PP2; iii) 20μM PP2; iv) 100μM PP2. **C.** Dose dependency of Src family kinase inhibitor PP2 at 240 sec after depolarisation. Curve fit, exponential fit. **D.** Glutamate release: i) Control 4AP; ii) 10μM PP2; iii) 10μM PP3. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to 37°C control at 440 sec (unpaired Student t-test). Data represent means ± SEM of experiments with three synaptosomal preparations (n=3).

produced a significant reduction in the 4AP-evoked release to 18.7 ± 1.8 nmol/mg, 13.6 ± 1.5 nmol/mg and 13.9 ± 0.9 nmol/mg with 10μM, 20μM and 100μM respectively (Figures 6.2B and 6.2C). Again, as a control, 4AP-evoked glutamate release was examined in the presence of 10μM PP2 and 10μM PP3. Results in fig. 6.2D reveal that the addition of PP2 reduces 4AP-evoked glutamate release to 13.3 ± 0.4 nmol/mg compared to control (20.5 ± 2.2 nmol/mg). This was in contrast to PP3 which did not significantly affect release evoked with 4AP (25.1 ± 3.2 nmol/mg). This suggests that the nRTK pathway involving the Src family kinases function in regulating depolarisation-induced glutamate release.

We next determined the effect of blocking SFKs on basal ERK 1&2 activities where synaptosomes were treated in the presence of 10μM PP2 or 10μM PP3 (Fig. 6.3A). Figure 6.3B shows that PP2 inhibited 32.6 ± 8.4 % of basal ERK 1&2 activation whilst PP3 produced a statistically non-significant effect. The fact that basal levels of ERK phosphorylation was reduced by 20% (a level similar to the 20% $Ca^{2+}$-dependency of basal ERK activation seen in chapter 4) appears to suggest that Src kinase nRTKs is involved in regulating $Ca^{2+}$-dependent ERK signalling during basal conditions.

Figures 6.4A and 6.4B demonstrate that 4AP-induced ERK 1&2 activation was also significantly attenuated by PP2 during depolarisation (4AP, 166.8 ± 13.6 %; PP2/4AP, 90.4 ± 5.3 %). In fact, treatment of synaptosomes with PP2 led to a complete inhibition of the depolarisation-induced component, indicating a strict requirement for SFKs in controlling ERK 1&2 signalling following membrane depolarisation.

As nRTKs were previously known to modulate $Ca^{2+}$-influx directly, we by-passed modulation at the level of VGCCs and synaptosomal excitability using ionomycin, so

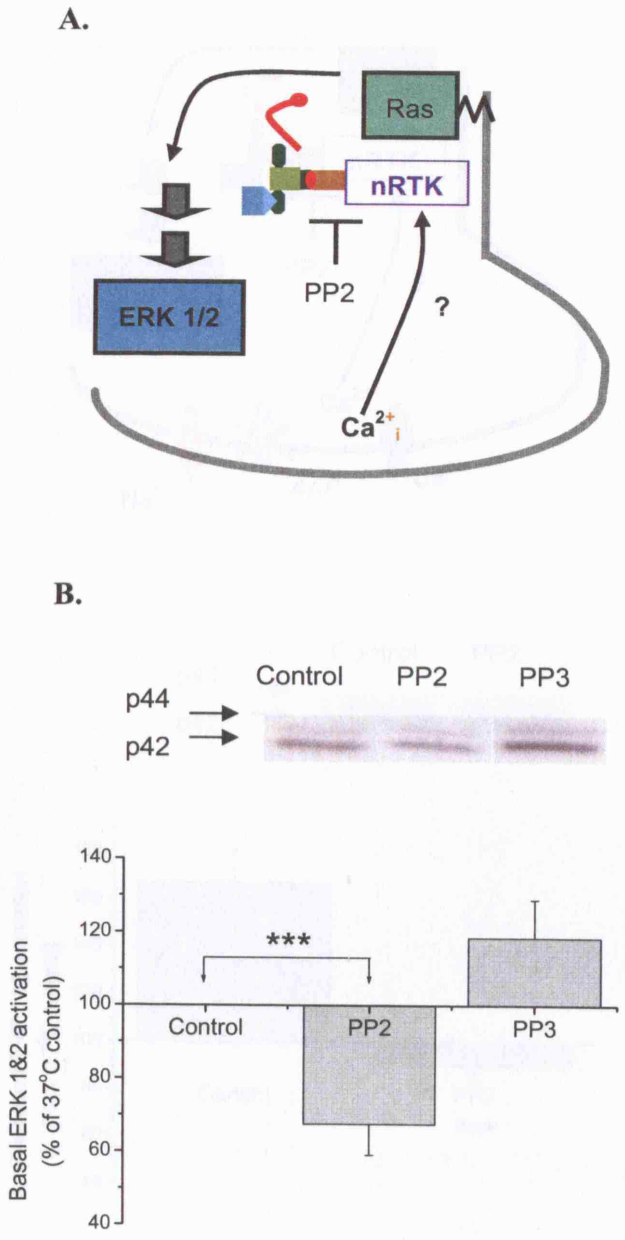



**Figure 6.3. Effect of PP2 and PP3 on basal ERK 1&2 activation.**

**A.** Scheme showing proposed effects of PP2 and PP3 on basal ERK 1&2 activation. **B.** Phosphoimages and quantification of basal phospho-ERK following treatment with PP2 or PP3. Synaptosomes (0.3 mg/ml) were preincubated in the absence (Control) or presence of 10μM PP2 (PP2) or 10μM PP3 (PP3) before the standard 10-minute incubation protocol. Samples separation and immunoblotting with phospho-p44/42 ERK (Thr202/Tyr204) antibody (NEB) was carried out as described in the Materials and Methods section. ***$p<0.001$ compared to 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with seven independent synaptosomal preparations (n=7).

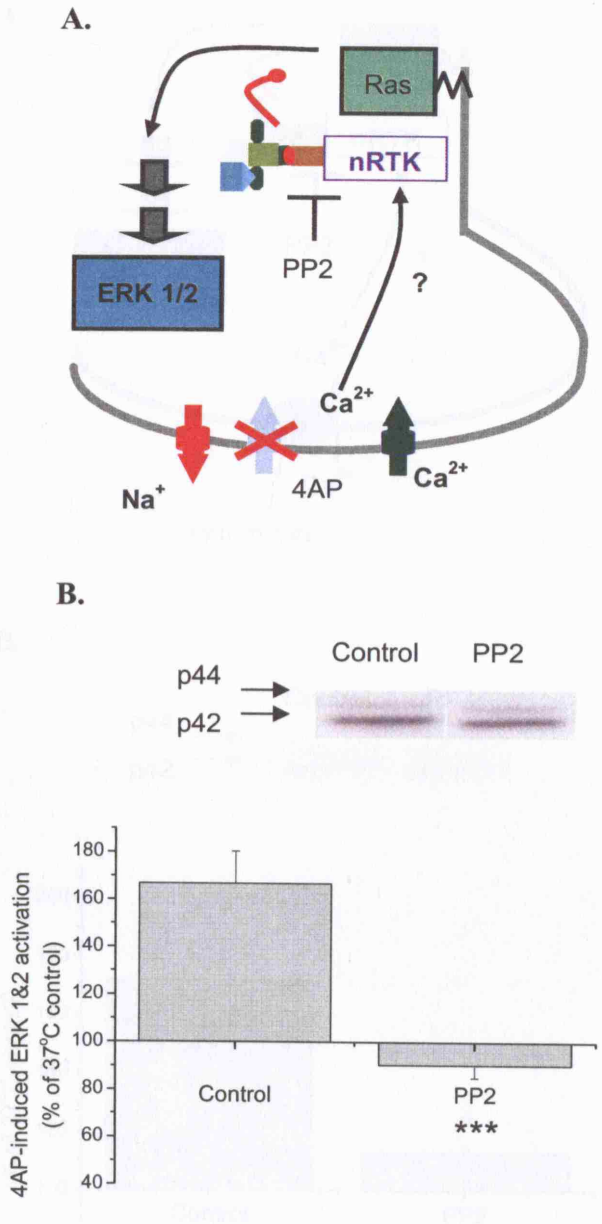



**Figure 6.4. Effect of PP2 on 4AP-induced ERK 1&2 activation.**

**A.** Scheme showing proposed effects of PP2 on 4AP-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of 4AP-induced phospho-ERK following treatment with or without PP2. Synaptosomes (0.3 mg/ml) were preincubated in the absence (Control) or presence of 10μM PP2 (PP2) before the standard 10-minute incubation protocol. ***$p<0.001$ compared to 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with seven independent synaptosomal preparations (n=7).

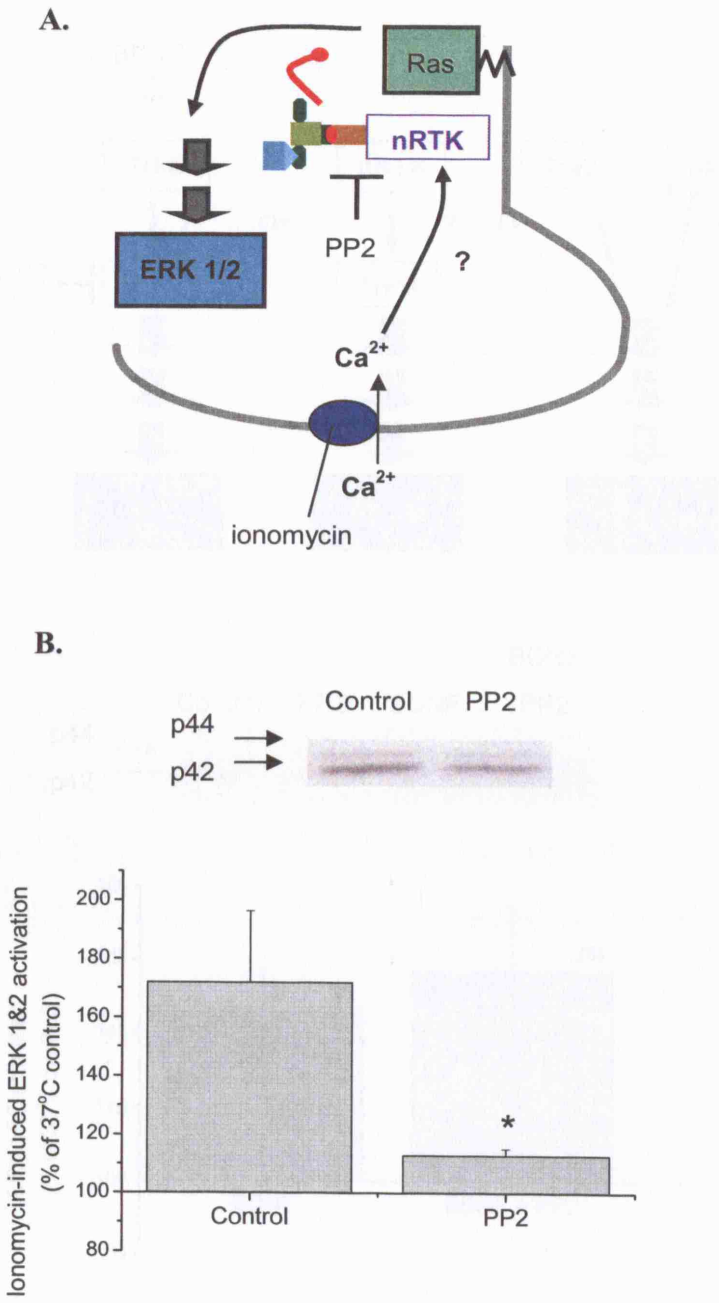



**Figure 6.5. Effect of PP2 on $Ca^{2+}$-activation of ERK 1&2 by direct $Ca^{2+}$ entry effected by ionophore ionomycin. A.** Scheme showing proposed effects of PP2 on ionomycin-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of ionomycin-induced phospho-ERK levels following treatment with or without PP2. Synaptosomes (0.3 mg/ml) were preincubated with vehicle (Control) or 10μM PP2 (PP2) before following the protocol measuring $Ca^{2+}$-dependency of ERK 1&2 activation with ionomycin, as described in the Materials and Methods section. *$p<0.05$ compared to 37°C ionomycin control (unpaired Student t-test). Data were means ± SEM of experiments studied with six independent synaptosomal preparations (n=6).

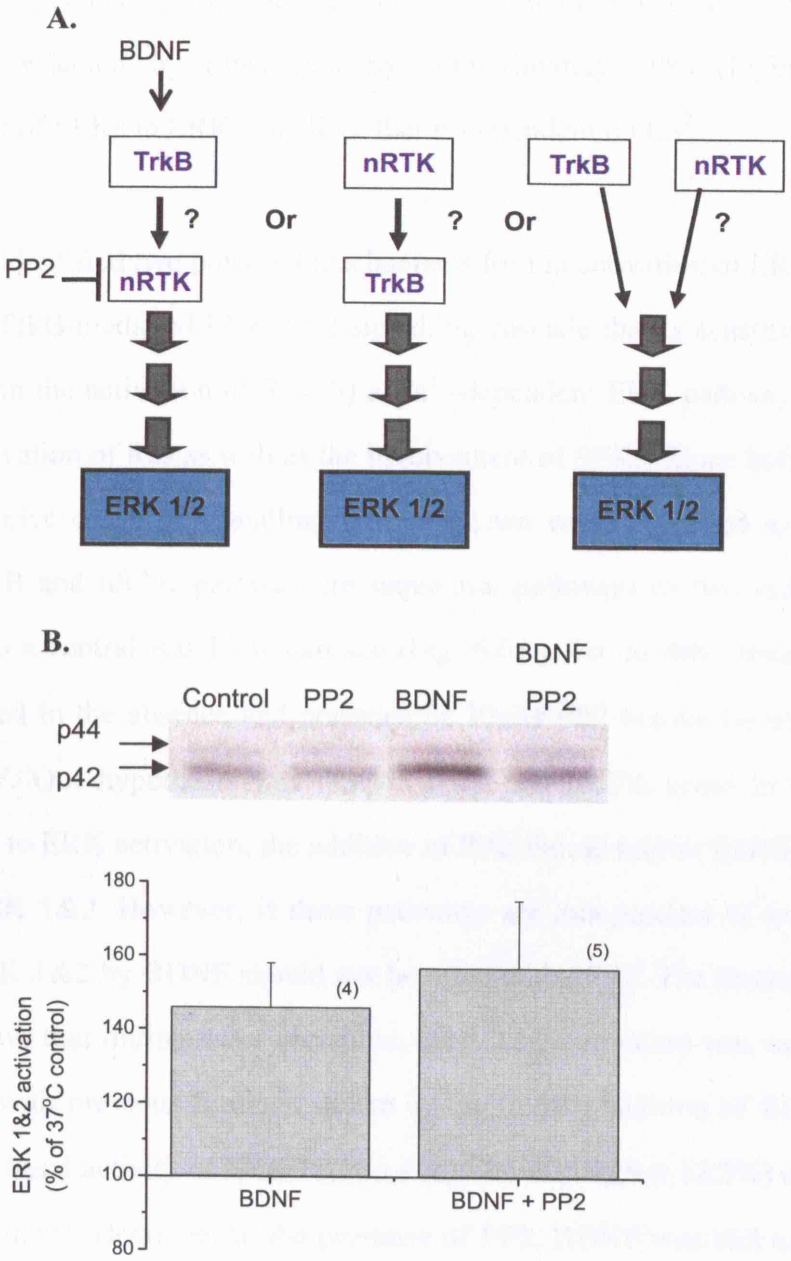



**Figure 6.6. TrkB and nRTK pathways are independent inputs that feed into the ERK signalling pathway. A.** Scheme showing proposed mechanisms of TrkB and nRTK pathways feeding into Ras-ERK cascade. **B.** Phosphoimages and quantification of phospho-ERK following BDNF and/or PP2 treatment. Synaptosomes (0.3 mg/ml) were preincubated with vehicle (Control) or 10μM PP2 (PP2) before the standard incubation protocol. Pellets were then resuspended and incubated in the absence (Control) or presence (BDNF) of 200ng/ml BDNF, either with (PP2) or without the addition of 10μM PP2. Levels of ERK 1&2 activation were presented as % from its respective controls. Data were means ± SEM of experiments studied with at least four independent synaptosomal preparations (n>4).

that any direct effects of SFKs on $Ca^{2+}$-dependent ERK 1&2 activation could be evaluated (Fig. 6.5A). Data show that PP2 significantly reduced $Ca^{2+}$-dependent ERK 1&2 activation effected by ionomycin by approximately 59% (Figure 6.5B), suggesting a role of SFKs in ERK signalling that is dependent on $Ca^{2+}$.

So far, we have identified two potential mechanisms for the activation of ERK 1&2 in this thesis: i) a TrkB-mediated ERK 1&2 signalling cascade that is sensitive to $Ca^{2+}$ and contingent on the activation of Ras; ii) a $Ca^{2+}$-dependent ERK pathway that also involves the activation of Ras as well as the involvement of SFKs. Since both of these mechanisms involve common signalling molecules, we were interested to examine whether the TrkB and nRTK pathway are sequential pathways or two independent inputs leading to a central Ras-ERK cascade (Fig. 6.6A). To do this, synaptosomes were preincubated in the absence and presence of 10μM PP2 before the addition of 200ng/ml BDNF. Our hypothesis was that, if TrkB and nRTK occur in the same pathway leading to ERK activation, the addition of PP2 should inhibit BDNF-induced activation of ERK 1&2. However, if these pathways are independent of each other, activation of ERK 1&2 by BDNF should not be affected by PP2. The immunoblot in Figure 6.6B shows that during basal condition, ERK 1&2 activation was reduced by PP2, consistent with previous findings shown in Fig. 6.3B. Addition of BDNF was able to stimulate basal activity of ERK 1&2 to a high level (145.9 ± 12.2%) compared to 100% 37°C control. However, in the presence of PP2, BDNF was still capable of stimulating basal ERK 1&2 activation by a similar amount (156.7 ± 18.5 %). This suggests that TrkB pathway does not involve SFKs and *vice versa*. Thus, the TrkB-mediated pathway and the $Ca^{2+}$-dependent pathway involving the actions of SFKs appear to be distinct inputs that converge to a common cascade leading to downstream ERK 1&2 activation.

## 6.4. Discussion

The non-receptor tyrosine kinases SFKs have been previously known to function as important regulators that mediate signals from cell surface receptors to stimulate a range of downstream signalling pathways including those of ERK 1&2 in many non-neuronal and neuronal preparations (Crossthwaite et al., 2004; Perkinton et al., 1999; Dikic et al., 1996; Finkbeiner and Greenberg, 1996; Rosen et al., 1994). This chapter aimed to study the role of SFKs in the regulation of ERK 1&2 activation and its relations with mechanisms underlying the control of neurotransmitter release in the presynaptic nerve terminal. Our data demonstrate that the inhibition of Src kinases with PP2 suppresses the depolarisation-induced glutamate release from cerebrocortical synaptosomes in a dose-dependent manner. We directly confirmed the involvement of SFKs in glutamate release using the inactive analogue of PP2, PP3. Results revealed that addition of 10μM PP2, but not PP3, reduced depolarisation-induced glutamate release. Thus, unlike studies by Ohnishi *et al.* showing a negative regulation of SFKs on dopamine release from neurons (Ohnishi et al., 2001), our data suggests a SFK-mediated pathway in effecting a facilitatory modulation of glutamate release in cerebrocortical synaptosomes. This is consistent with a number of studies which also demonstrated a positive modulatory role of SFKs in neurotransmitter release (Wang, 2003; Evans and Pocock, 1999) . In addition, we showed that PP2 affects basal (unstimulated) glutamate release in a dose-dependent manner where it is likely to have non-specific effects at higher concentrations. Indeed, this question of selectivity of PP2 had been raised previously (Bain et al., 2003). It is possible that the inhibition of glutamate release at high concentrations of PP2 is due to the drug depleting the readily releasable pool of SVs as a result of the release obtained in the absence of any secretagogues (Fig. 6.1), although further experiments are warranted to determine whether this is the case.

Our data, thus far, have shown that ERK 1&2 signalling might be a possible route

through which SFKs facilitate neurotransmitter release. Results presented here suggested a crosstalk between the nRTK pathway and the ERK 1&2 activation pathway where application of PP2, but not PP3, significantly attenuated both basal and depolarisation-induced ERK 1&2 activation. Unlike studies in the hippocampal slices where ERK and SFK activities were in separate cellular compartments (Corvol et al., 2005), the data in this chapter suggest that SFKs and ERK 1&2 occur in the same nerve terminal and that they cross-talk with each other to affect presynaptic functions. This is perhaps not surprising as we have shown earlier in this thesis (see chapter 4) that basal and depolarisation-induced activation of ERK 1&2 can be mediated through a $Ca^{2+}$-dependent mechanism and that the nRTKs have been previously known also to be directly or indirectly $Ca^{2+}$-dependent (Siciliano et al., 1996). Indeed, several reports have implicated a role for SFKs in depolarisation-induced ERK signalling where SFKs are activated following $Ca^{2+}$ influx (Dikic et al., 1996; Lev et al., 1995; Rusanescu et al., 1995).

Upon activation by $Ca^{2+}$, SFKs might act to affect basal and depolarisation-induced activation of ERK 1&2 by modulating the function of VGCC to affect $Ca^{2+}$ influx. This has been demonstrated by reports showing that PP2 can reduce voltage-dependent $Ca^{2+}$ influx (Wang, 2003; Evans and Pocock, 1999). In addition, SFKs may also regulate $Ca^{2+}$-dependent ERK 1&2 activation by modulating the activities of other voltage-gated ion channels including $Na^{+}$ channels (Ratcliffe et al., 2000) or potassium channels (Fadool et al., 1997). To eliminate these possibilities of SFK modulation at the level of synaptosomal excitability and VGCC modulation, we bypassed these mechanisms by mediating direct $Ca^{2+}$ entry using the $Ca^{2+}$ ionophore ionomycin. Our data, indeed, demonstrated the role of SFKs in $Ca^{2+}$-dependent ERK signalling downstream of $Ca^{2+}$ entry and membrane excitability. This was apparent as ionomycin-induced ERK 1&2 activation was strongly inhibited by PP2. This result is also comparable with studies by other groups where Pyk2 has been shown to have a role in ionomycin-induced activation of ERK (Ginnan and Singer, 2002). One way by

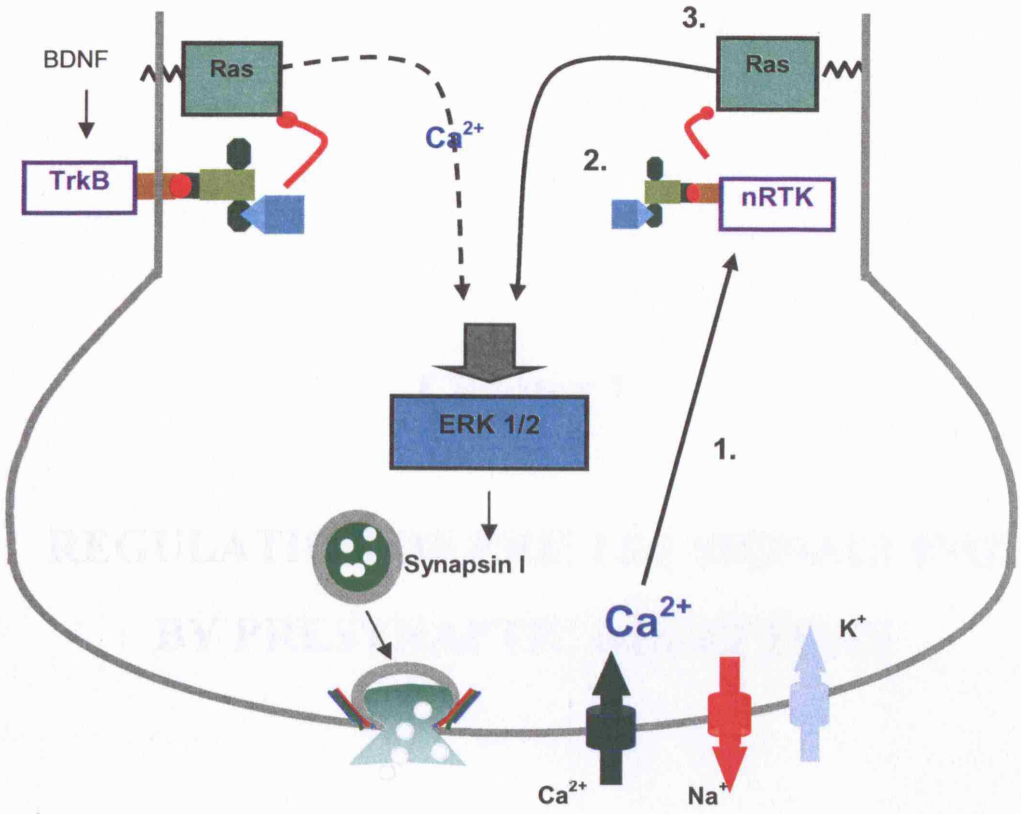
which SFKs may act downstream of membrane excitability and VGCC is by acting as scaffold proteins that recruit regulators of the ERK 1&2 cascade, in so doing, bring about ERK 1&2 activation more effectively. This is supported by previous studies which have shown that stimulation of cell surface receptors lead to the formation of signalling complex involving SFKs and adaptors such as Shc and Grb2 before ERK activation (Luttrell et al., 1999). Furthermore, SFKs may also interact with other synaptic proteins in the release machinery (Foster-Barber and Bishop, 1998; Onofri et al., 1997; Barnekow et al., 1990) to then activate signalling of ERK 1&2.

In addition, activation of SFKs may initiate the activation of regulators such as Ras to cause subsequent downstream ERK 1&2 signalling (Kalia et al., 2004). Indeed, in chapter 4, we have shown that depolarisation-induced ERK 1&2 was dependent on Ras activation. Given that we have now shown that depolarisation-induced ERK 1&2 was also dependent on SFKs, this might suggest that SFKs act upstream or at the level of Ras. Further experiments will be needed to directly address the mechanisms by which SFKs may act to regulate ERK 1&2 signalling.

Our data have, thus far, identified two potential mechanisms for ERK 1&2 activation, one being the TrkB-Ras mediated pathway that is sensitive to $Ca^{2+}$, and the other, a Ras and $Ca^{2+}$-dependent ERK 1&2 cascade that involves the actions of SFKs. One immediate question arises as to whether there is cross-talk between these two pathways. The foregoing chapters have shown that both the Trk-mediated and nRTK-mediated pathways appear to lead to a common Ras-ERK cascade that is contingent on $Ca^{2+}$, suggesting that the two cascades impinge upon each other leading to subsequent activation of ERK 1&2. Thus, an interest was to examine whether they are sequential pathways that followed one another or separate inputs that impinge on ERK 1&2 at a common level. The results in this chapter revealed that when BDNF was added to stimulate TrkB-mediated ERK phosphorylation in the presence of PP2, the effect of BDNF on ERK 1&2 activation persisted. This appears to suggest that

TrkB and nRTK-mediated ERK signalling pathways are two distinct inputs where TrkB activation is not upstream of nRTK leading to ERK activation and *vice versa.*

The foregoing finding is in agreement with our previous results in chapter 4 where we have shown that depolarisation did not further potentiate the facilitatory effect of BDNF on ERK 1&2 activation, thereby suggesting that the TrkB and depolarisation-induced pathways are independent inputs. Thus, our results seem to indicate that nerve terminal depolarisation can result in SFK-mediated activation of ERK 1&2 signalling independently of a TrkB-mediated pathway with both of these inputs later converging to cause ERK activation by feeding into a common Ras-dependent pathway **(Schematic 6.4)**. Taken together, these data give us a better insight into the complexity of the cross-talking between presynaptic signalling pathways in the nerve terminal and the precise ways by which ERK 1&2 activation can be modulated differently in response to specific stimuli.




**Schematic 6.4. The potential role of SFKs in the $Ca^{2+}$-dependent activation of ERK 1&2, independently of the BDNF-mediated cascade.** 1.) SFKs are activated by $Ca^{2+}$; 2.) Activated SFKs may recruit upstream ERK 1&2 mediators such as Shc and Grb2 to facilitate ERK signalling; 3.) SFKs may initiate the activation of Ras, leading to downstream ERK 1&2 activation.

# Chapter 7

# REGULATION OF ERK 1&2 SIGNALLING BY PRESYNAPTIC RECEPTORS

## 7.1. Introduction

The ability of neurons to grade synaptic responses has been shown to be a result of the modulation and the cross-talk between many molecular signalling pathways, involving the activation of inhibitory and facilitatory presynaptic receptors. γ-aminobutyric acid (GABA) has been known as the major inhibitory neurotransmitter in the brain by acting through $GABA_A$, $GABA_B$ and $GABA_C$ receptors. Whilst $GABA_A$ and $GABA_C$ receptors have been primarily implicated in fast inhibitory neurotransmission by increasing $Cl^-$ channel activities (Kullmann et al., 2005; Stephenson, 1988), inhibitory effects produced by $GABA_B$ receptors are mediated through the activation of $G_{i/o}$-proteins (Bowery et al., 2002; Odagaki and Koyama, 2001; Bowery, 1989).

Studies have shown that $GABA_B$ receptors are highly localised in the cortex (Charles et al., 2003) where they function both pre- and post-synaptically to suppress synaptic transmission (Kaneda and Kita, 2005). In both cerebral cortical slices and synaptosomal preparations, the activation of presynaptic $GABA_B$ receptors has been demonstrated to result in the inhibition of stimulus-evoked glutamate release (Perkinton and Sihra, 1998; Pende et al., 1993; Bonanno and Raiteri, 1992; Potashner, 1979). These inhibitory effects of $GABA_B$ receptor and $G_{i/o}$ protein activation have been shown to be mediated through several mechanisms, including the inhibition of $Ca^{2+}$ influx through P/Q and N-type VGCCs (Perkinton and Sihra, 1998; Dittman and Regehr, 1996), the activation of Kv channels (Kubota et al., 2003; Misgeld et al., 1989), and the reduction of adenylyl cyclase (AC) activity and cAMP levels leading to a decrease in SV recruitment (Fairfax et al., 2004; Sakaba and Neher, 2003).

In several systems, $GABA_B$ receptor-mediated inhibitory function has been shown to interact with signalling cascades involving protein kinases such as PKA and PKC

(Kubota et al., 2003; Perkinton and Sihra, 1998). These studies have demonstrated that $GABA_B$-mediated reduction of neurotransmitter release through the activation of $K^+$ channels and/or the inhibition of VGCCs is negatively regulated by PKA and PKC (Kubota et al., 2003; Perkinton and Sihra, 1998). Apart from crosstalking with the PKA and PKC signalling pathways, studies have also demonstrated a link between the $GABA_B$-mediated pathway and the ERK activation cascade. Experiments in the mouse cortex and hippocampus have revealed that activation of $GABA_B$ receptors is associated with a decrease in ERK phosphorylation, where γ-hydroxybutyrate (GHB), a natural derivative of GABA, was able to reduce the activation of ERK, an effect which could be mimicked by the $GABA_B$ agonist baclofen (Ren and Mody, 2003). However, this was contradicted by studies showing that ERK phosphorylation is increased following baclofen addition (Vanhoose et al., 2002). Furthermore, recruitment of Src kinases and the Ras complex has been shown to occur upon $GABA_B$ receptor activation and this is typically associated with modulation of VGCC activities as well as an increase in ERK activation ((Richman et al., 2004), see chapter 6).

This chapter focuses on characterising the role of the $GABA_B$ receptor type in the nerve terminal, in particular, its involvement in the regulation of ERK 1&2 activation. We aimed to determine whether $GABA_B$-induced pathway might provide a negative regulation of ERK phosphorylation/activation and, if so, how might this $GABA_BR$-coupled ERK signalling be mediated. In addition, we examined whether membrane depolarisation and the activation of TrkB receptors can affect the coupling of ERK 1&2 and $GABA_B$ receptors.

## 7.2. Materials and Methods

### 7.2.1. Synaptosomal preparation

Synaptosomes were prepared as described in section 2.1.1.

### 7.2.2. Cytosolic $Ca^{2+}$ measurements using fura-2

Cytosolic $Ca^{2+}$ was measured with the ratiometric $Ca^{2+}$ indicator, fura-2, using on-line fluorimetry as described in Section 2.3. Synaptosomes were preincubated at 37°C in HBM containing 0.1mM $CaCl_2$ and 1mg/ml BSA and loaded with fura-2-AM (5μM) for 20 min. Fura-loaded synaptosomes were then centrifuged and the supernatant discarded. Synaptosomal pellets were resuspended in HBM containing 1mg/ml BSA and incubated in the spectrofluorimeter (Perkin-Elmer, Emeryville, CA) with $CaCl_2$ (1mM) added after 3 min. Depolarisation was achieved with the addition of the secretagogues, 4AP (1mM) or KCl (10mM), at 10 min. BDNF (200ng/ml) or PD98059 (50μM) was added at the start of the incubation and baclofen (50μM) was added after 8 min. Excitation wavelengths of 340 and 380nm and emission wavelength of 505nm were used and data were collected at 3.5 sec intervals. Calibration procedures were carried out with 0.1% SDS and 5mM Na-EGTA as described in Section 2.3.

### 7.2.3. Standard incubation for ERK 1&2 activation/phosphorylation

Synaptosomes were resuspended in HBM containing 1mg/ml BSA and incubated at 37°C as described in the standard incubation protocol indicated in section 2.4.1. $CaCl_2$ (1mM) was then added at 3 min followed by the addition of 4AP (1mM) at 10 min. BDNF (200ng/ml), GABase (0.6 Units) or PD98059 (50μM) was added at the start of incubation and baclofen (50μM) was added after 8 min. Reactions were terminated with the addition of sample buffer 1 min after the addition of secretagogue.

### 7.2.4. SDS-PAGE and Immunoblotting

Proteins were separated on SDS-PAGE and subjected to immunoblotting as described in sections 2.4.5 and 2.4.6.

### 7.2.5. Statistical analysis

Student's paired t-test was used to assess the confidence levels of significant differences between two sets of data.

### 7.2.6. Reagents

Stock solution of BDNF (1mg/ml) was obtained in buffer containing PBS after which a working solution of 20μg/ml was made using water. BDNF was used at final concentration of 200ng/ml.

A working solution of baclofen (5mM) was made using water. Baclofen was used at final concentration of 50μM.

GABase was used at a concentration with enzyme activity of 3 Units/ml.

A 50mM stock solution of PD98059 was obtained using DMSO, which was then further diluted to 5mM with HBM/BSA as a working solution. PD98059 was used at 50μM as a final concentration.

## 7.3. Results

To determine the role of $GABA_B$ receptor activation on ERK 1&2 activation, synaptosomes were incubated in the presence of 50μM (-)-baclofen, a $GABA_B$ receptor agonist (Bowery, 1989) (Fig. 7.1A). Figure 7.1B shows that the addition of baclofen did not significantly affect basal levels of ERK 1&2 activation (baclofen, 102.6 ± 3.2 % compared to 100% 37°C control). This might suggest that $GABA_B$ receptors do not play a part in modulating basal ERK 1&2 activities. However, it is possible that this lack of effect of baclofen was due to the presence of endogenous GABA released into the medium which is capable of stimulating the presynaptic $GABA_B$ receptors and thereby eliminating any effect of exogenous agonist. Thus, to clarify the role of $GABA_B$ receptor in modulating presynaptic ERK 1&2, we used exogenous GABase, an enzyme that catalyses the breakdown of GABA, to determine whether basal activation of ERK 1&2 were affected in the absence of putative endogenous GABA-mediated inhibition (Fig. 7.1C). Results show that basal ERK 1&2 activation was significantly increased by 18.8 ± 6.7 % above control levels in GABase-treated synaptosomes, indicating that elimination of inhibition mediated by GABA receptor activation can lead to the elevation of 37°C/basal activation of ERK 1&2. When synaptosomes were treated with baclofen in the presence of GABase, baclofen was able to clearly attenuate the level of basal ERK 1&2 activities to 106.6 ± 4.8 % compared to conditions in the absence of baclofen (118.8 ± 6.8 %). Together, these data suggest that $GABA_B$ receptors are present in the nerve terminals and are involved in the negative modulation of presynaptic ERK 1&2 activities during basal conditions.

We next examined the effect of $GABA_B$ action on depolarisation/$Ca^{2+}$-dependent ERK 1&2 activation (Fig. 7.2A). Figure 7.2B illustrates that depolarisation-induced ERK 1&2 activation was sensitive to the addition of baclofen. Data revealed that in

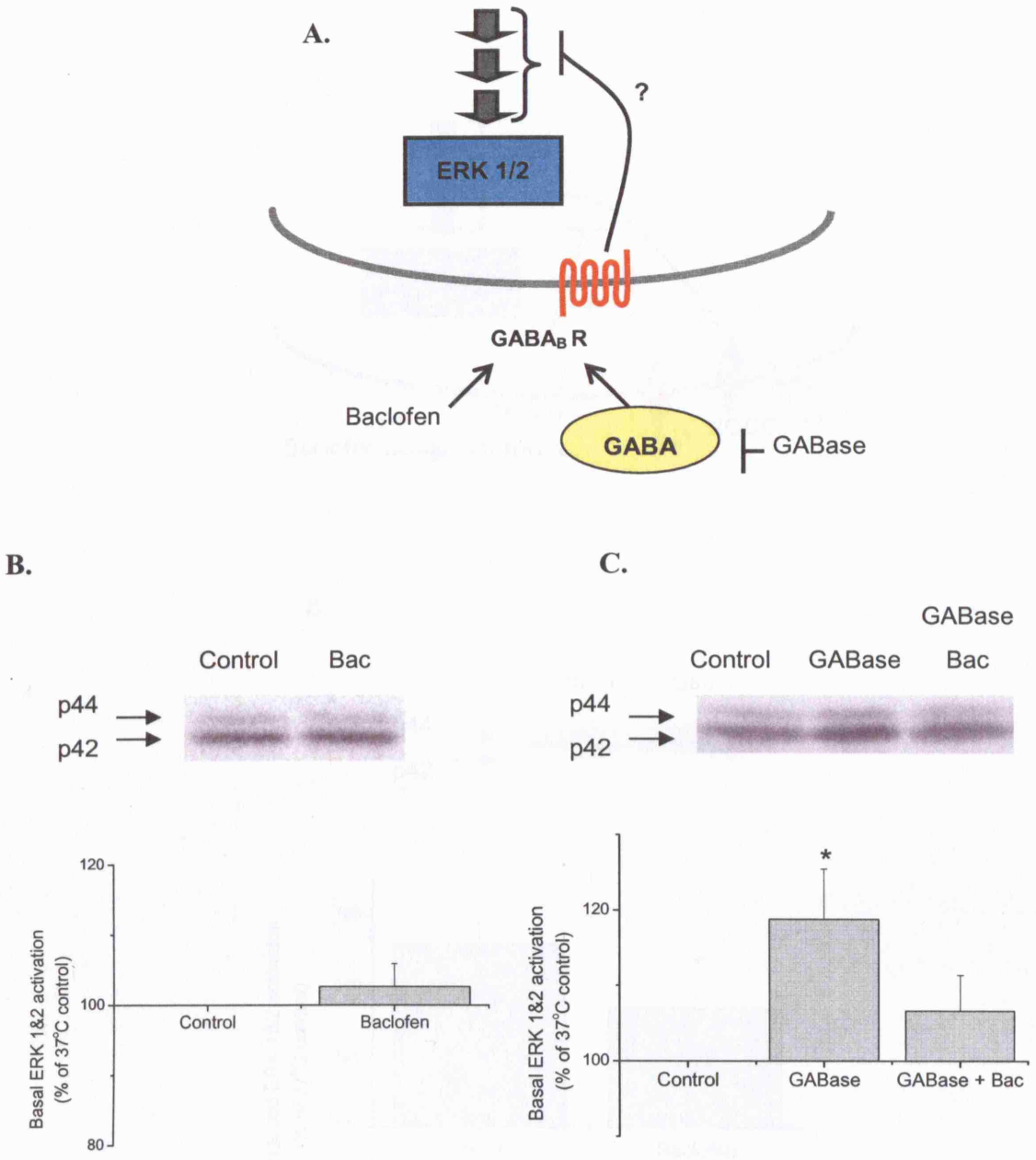



**Figure 7.1. Effect of baclofen, a GABA$_B$R agonist and/or GABase on basal ERK 1&2 activation.** **A.** Scheme showing proposed effects of baclofen and GABase on basal ERK 1&2 activation. **B.** Phosphoimages and quantification of basal phospho-ERK levels with or without treatment of baclofen. Synaptosomes (0.3 mg/ml) were incubated in the absence (Control) and presence (Baclofen) of 50μM baclofen. Reaction was then terminated and samples separation and immunoblotting with phospho-p44/42 ERK (Thr202/Tyr204) antibody (NEB) was carried out as described in the Materials and Methods section. Data represent means ± SEM of experiments carried out with five independent synaptosomal preparations (n=5). **C.** Phosphoimages and quantification of basal phospho-ERK levels with or without treatment of GABase (0.6U) and/or baclofen. *p<0.05 GABase compared to 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with three independent synaptosomal preparations (n=3).

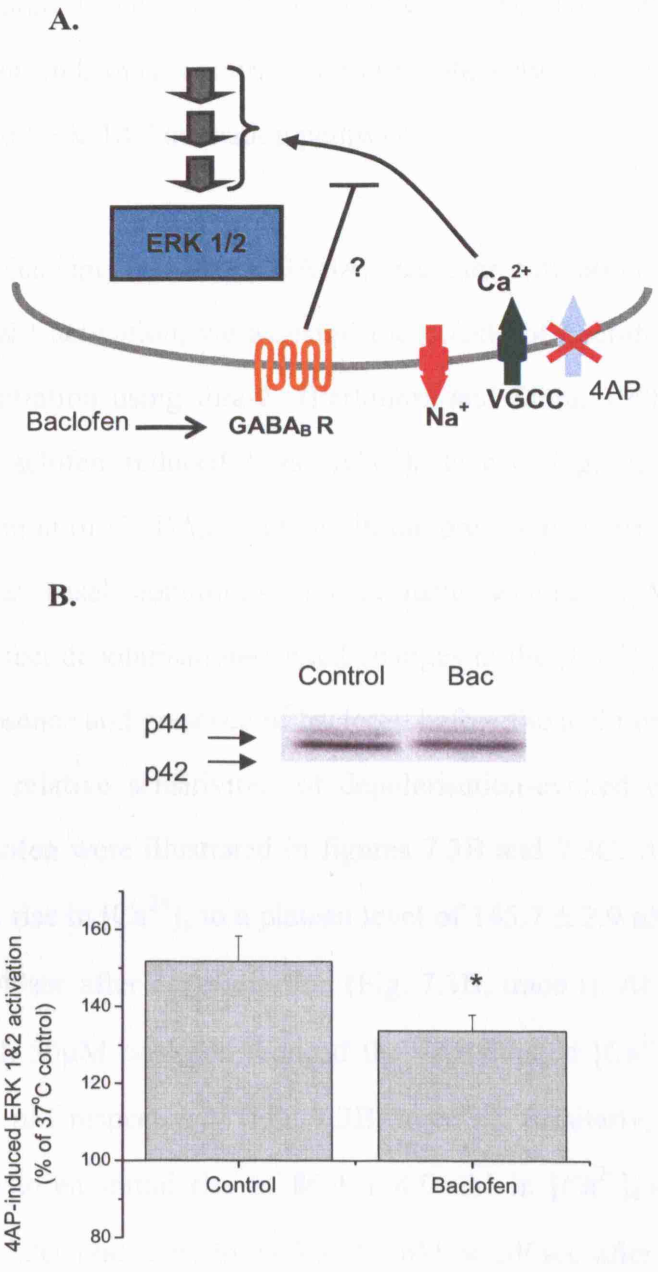



**Figure 7.2. Effect of Baclofen, a $GABA_BR$ agonist, on depolarisation-induced ERK 1&2 activation.** **A.** Scheme showing proposed effects of baclofen on 4AP-induced ERK 1&2 activation. **B.** Phosphoimages and quantification of 4AP-induced phospho-ERK levels following treatment with or without baclofen. Synaptosomes (0.3 mg/ml) were incubated with (Baclofen) or without (Control) the addition of 50μM baclofen at 8 min. *$p<0.05$ compared to 4AP control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with four independent synaptosomal preparations (n=4).

the presence of baclofen, 4AP-evoked activation of ERK 1&2 was suppressed to 133.8 ± 4.2 % compared to the 151.7 ± 6.6 % 37°C control. This 18% inhibition is statistically significant and, thus, confirms the crosstalk between $GABA_B$ receptor-mediated pathway and ERK 1&2 activation pathway.

To examine the mechanisms by which $GABA_B$ receptor activation transduces the inhibition of ERK 1&2 activation, we assessed the effects of baclofen on cytosolic $Ca^{2+}$ ($[Ca^{2+}]_c$) concentration using fura-2 (Perkinton and Sihra, 1998) (Fig. 7.3A). Results show that baclofen reduced basal $[Ca^{2+}]_c$ levels (Fig. 7.3B and 7.3C), suggesting that activation of $GABA_B$ receptors in the presynaptic terminal can affect $Ca^{2+}$ dynamics under basal conditions. To evaluate whether $GABA_B$ receptor activation can also affect depolarisation-evoked changes in the $[Ca^{2+}]_c$, synaptosomes were treated in the absence and presence of baclofen before the addition of 1mM 4AP or 10mM KCl. The relative sensitivities of depolarisation-evoked changes in the $[Ca^{2+}]_c$ levels to baclofen were illustrated in figures 7.3B and 7.3C. As can be seen, 4AP (1mM) caused a rise in $[Ca^{2+}]_c$ to a plateau level of 145.7 ± 2.9 nM at 40sec and 169.6 ± 4.6 nM at 240sec after depolarisation (Fig. 7.3B, trace i). At the same time points, treatment with 50μM baclofen reduced the 4AP-evoked $[Ca^{2+}]_c$ increase by about 16 nM and 15 nM respectively (Fig. 7.3B, trace ii). Similarly, depolarisation with 10mM KCl led to an initial rise of 86.4 ± 4.0 nM in $[Ca^{2+}]_c$ at 40 sec after depolarisation which later plateaued to 74.3 ± 7.3 nM at 240sec after depolarisation (Fig. 7.3C, trace i). This KCl-evoked $[Ca^{2+}]_c$ was inhibited to 57.8 ± 5.2 nM and 47.2 ± 4.3 nM at 40 sec and 240 sec after depolarisation respectively following the addition of baclofen (Fig. 7.3C, trace ii). While these data suggests that baclofen was able to cause some reduction in basal $[Ca^{2+}]_c$, it is not clear whether the $GABA_B$-mediated inhibition of depolarisation-induced $Ca^{2+}$ influx is a reflection of the inhibitory effect of baclofen on basal $[Ca^{2+}]_c$. Thus, further studies using statistical methods such as 2-way ANOVA, taking into account time and condition variables, may be useful to

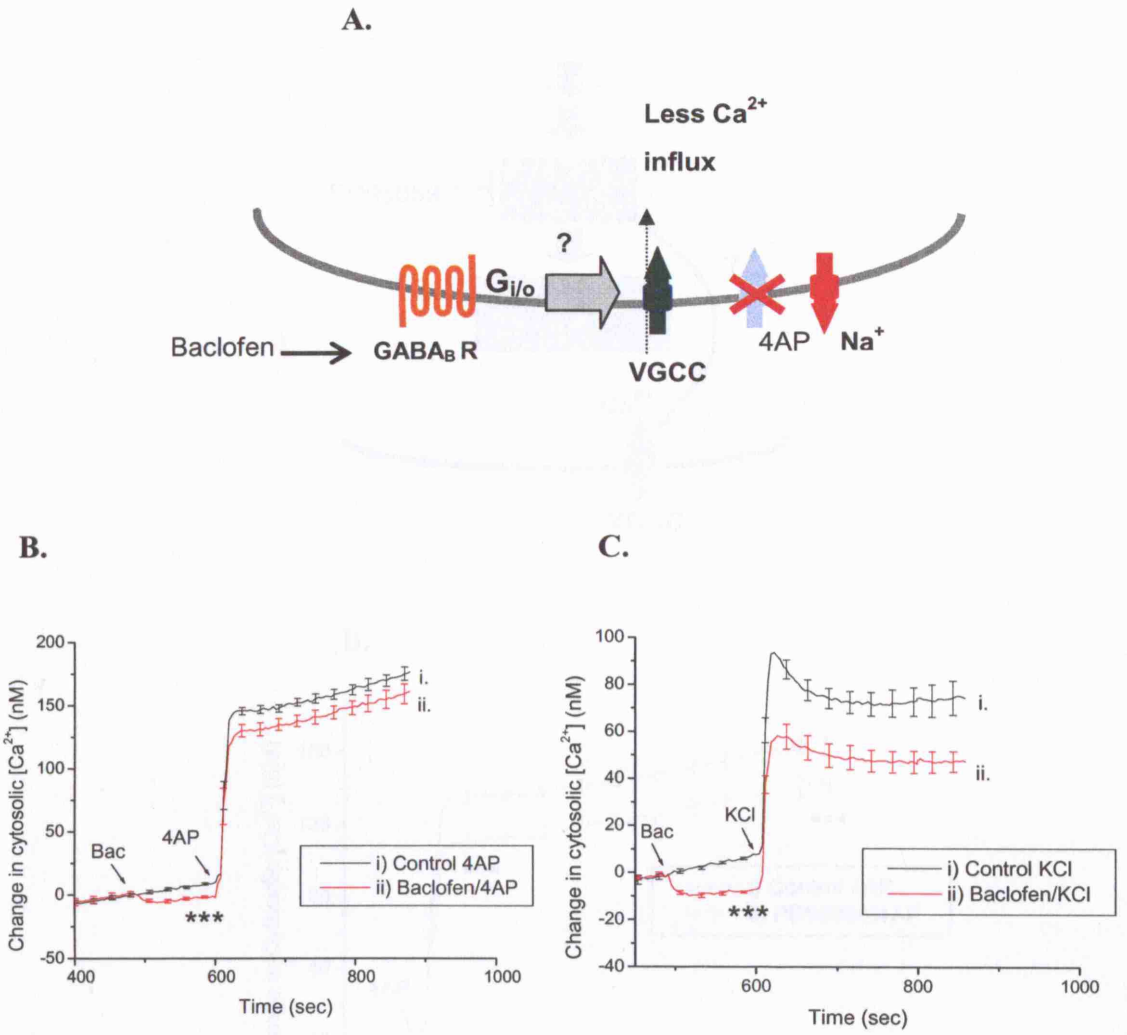



**Figure 7.3. Effect of baclofen on basal intracellular $Ca^{2+}$ and depolarisation-induced $Ca^{2+}$ influx.** **A.** Scheme showing proposed effects of baclofen on intracellular $Ca^{2+}$. **B.** Fura trace for 4AP-induced changes of cytosolic $Ca^{2+}$ concentrations. Synaptosomes were loaded with fura-2-AM as indicated in the Materials and Methods section. Pellet was then resuspended and incubated in the absence (Control) or presence (Baclofen) of 50μM baclofen, added at 8 min. Depolarisation was achieved with addition of 1mM 4AP at 10 min. Change in cytosolic $[Ca^{2+}]$ was calculated from the point of baclofen addition (480 sec). Data represent means ± SEM of experiments carried out with nine (n=9) and six (n=6) independent synaptosomal preparations for control 4AP and baclofen/4AP samples, respectively. **C.** Fura trace for basal and 10mM KCl-induced changes of cytosolic $Ca^{2+}$ concentrations. Data represent means ± SEM of experiments carried out with eleven (n=11) and six (n=6) independent synaptosomal preparations for control KCl and baclofen/KCl samples, respectively. ***$p<0.001$ compared to basal control (unpaired Student t-test).

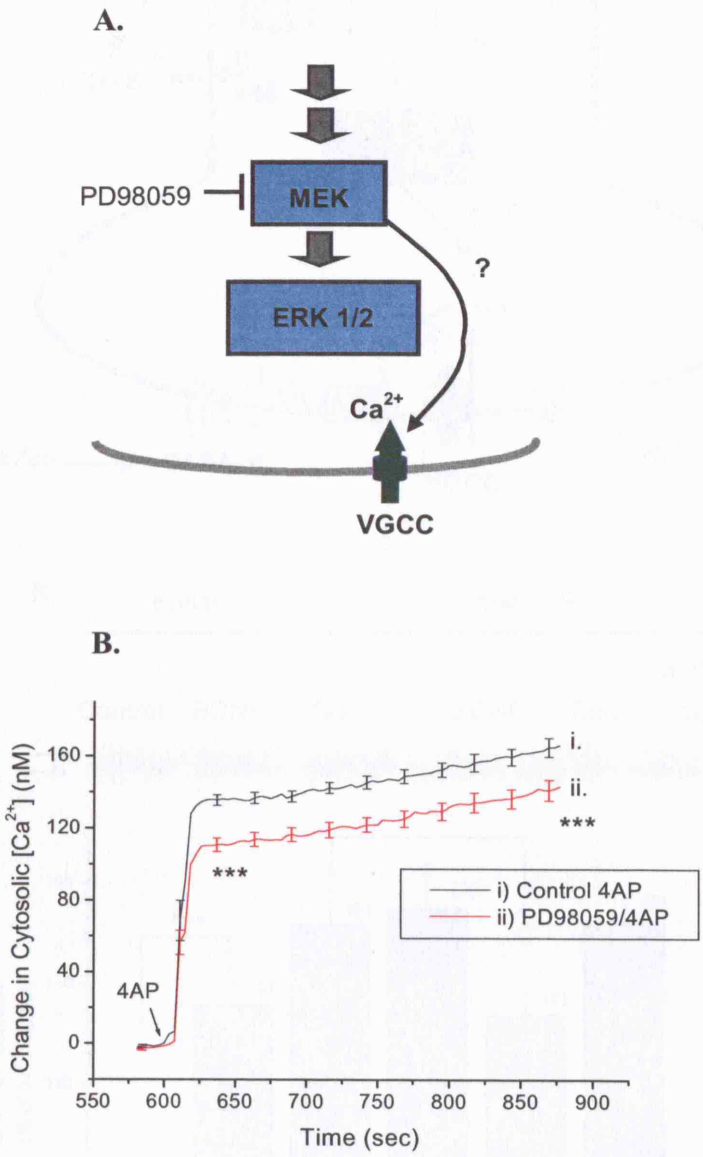



**Figure 7.4. Effect of PD98059, a MEK inhibitor, on intracellular $Ca^{2+}$.**

**A.** Scheme showing proposed effects of PD98059 on intracellular $Ca^{2+}$ levels. **B.** Fura trace of effects on 4AP-induced changes of cytosolic $Ca^{2+}$ concentrations. Synaptosomes were loaded with fura-2-AM as indicated in the Materials and Methods section. Pellet was then resuspended and incubated in the absence (Control) or presence (PD98059) of 50μM PD98059. Depolarisation was achieved with addition of 1mM 4AP at 10 min. ***$p<0.001$ compared to 37°C control (unpaired Student t-test). Data represent means ± SEM of experiments carried out with nine (n=9) and seven (n=7) independent synaptosomal preparations for control and PD98059 respectively.

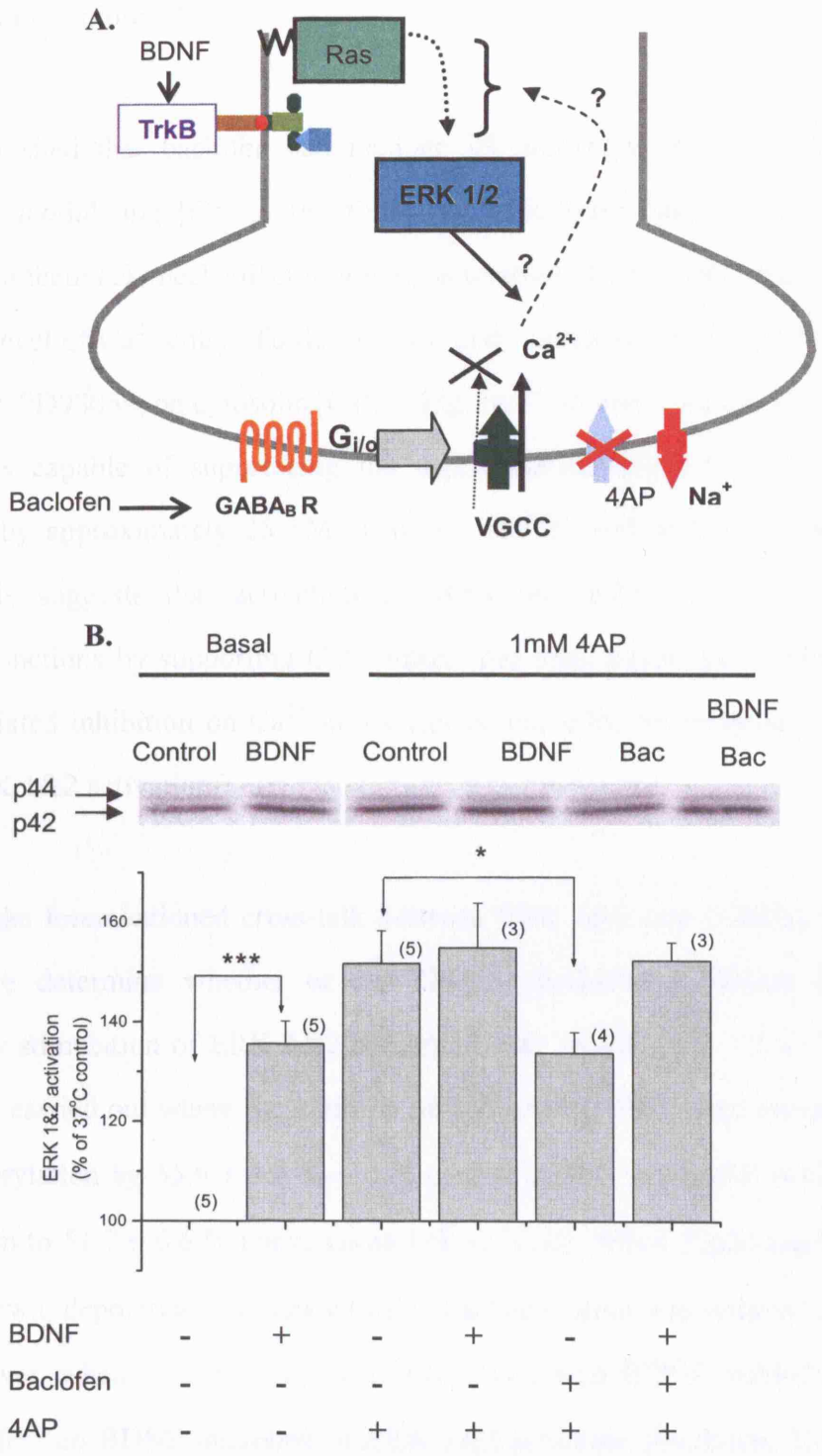



**Figure 7.5. Effect of BDNF and baclofen on 4AP-induced ERK 1&2 activation.**

**A.** Scheme showing proposed effects of BDNF and baclofen on depolarisation-induced activation of ERK. **B.** Phosphoimages and quantification of 4AP-induced phospho-ERK levels. Synaptosomes (0.3 mg/ml) were incubated 200ng/ml BDNF. Vehicle (Control) or 50μM baclofen (Baclofen) was added at 8 min. Synaptosomes were depolarised with 1mM 4AP and reaction was terminated. *$p<0.05$ compared to 4AP control, ***$p<0.001$ compared to basal control (unpaired Student t-test). Data represent means ± SEM of three or more ($n\geq3$) independent synaptosomal preparations as indicated.

evaluate this more rigorously.

Having established that baclofen can mediate its inhibitory effect on ERK 1&2 activation by modulating $[Ca^{2+}]_c$, the following experiment aimed to explore the possibility of a feedback mechanism following activation of ERK 1&2 that might also occur at the level of $Ca^{2+}$ entry. To do this, we first looked at the effect of the MEK 1&2 inhibitor, PD98059, on cytosolic $[Ca^{2+}]_c$. Figures 7.4A and 7.4B demonstrate that PD98059 was capable of suppressing the depolarisation-induced $[Ca^{2+}]_c$ increase significantly by approximately 25 nM at 40 sec and 23 nM at 240 sec after 4AP addition. This suggests that activation of MEK and ERK 1&2 can modulate presynaptic functions by supporting $Ca^{2+}$ influx, and thus, poses the possibility that $GABA_B$-mediated inhibition on $Ca^{2+}$ influx can be occluded by modulation of $Ca^{2+}$ influx by ERK 1&2 activation.

To examine the forementioned cross-talk between ERK 1&2 and $GABA_B$-mediated signalling, we determine whether or not $GABA_B$-mediated inhibition could be overridden by stimulation of ERK 1&2 activation with BDNF (Fig. 7.5A). Relevant controls were carried out where the addition of 200ng/ml BDNF alone elevated ERK 1&2 phosphorylation by 35.6 ± 4.6 % above control whilst 1mM 4AP evoked ERK 1&2 activation to 51.7 ± 6.6 % above control (Fig. 7.5B). When 50μM baclofen was added on its own, depolarisation-induced ERK 1&2 activation was reduced by 18.0 ± 4.2 %. However, when synaptosomes were stimulated with BDNF, baclofen had no significant effect on BDNF-induction of ERK 1&2 activities (Baclofen, 133.8 ± 4.2 %; BDNF/baclofen, 152.4 ± 3.7 %). This suggests that the $GABA_B$ receptor-mediated inhibition of ERK 1&2 activation can be overcome with the addition of BDNF, thus, implicating a cross-talk mechanism between the excitatory TrkB and the inhibitory $GABA_B$ receptor–mediated cascades.

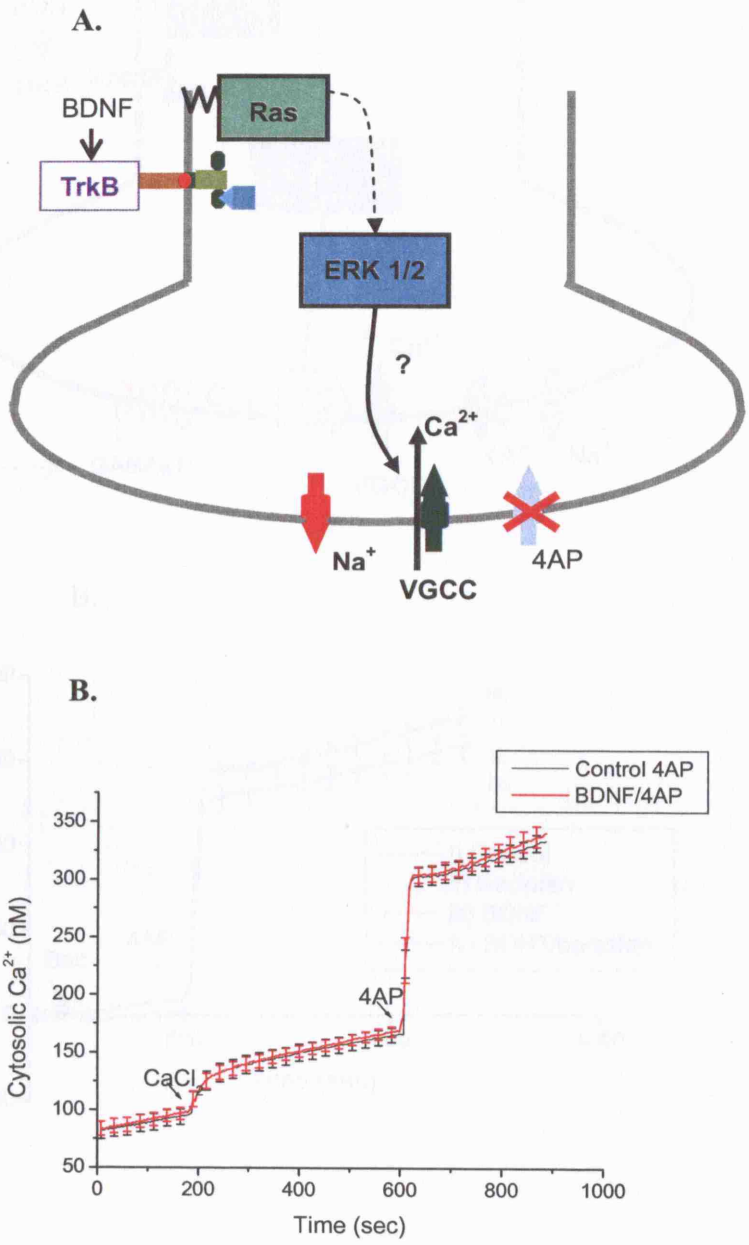



**Figure 7.6. Effect of BDNF on $Ca^{2+}$ influx induced by secretagogue 4AP.**

**A.** Scheme showing proposed effects of BDNF on 4AP-induced $Ca^{2+}$ influx. **B.** Fura trace for effects on 4AP-induced changes of cytosolic $Ca^{2+}$ concentrations. Synaptosomes were loaded with fura-2-AM as indicated in the Materials and Methods section. Pellet was resuspended and incubated in the absence (Control) or presence (BDNF) of 200ng/ml BDNF. Depolarisation was achieved with addition of 1mM 4AP at 10 min. Data represent means ± SEM of experiments carried out with nine (n=9) and eight (n=8) independent synaptosomal preparations for control and BDNF-treated samples respectively.

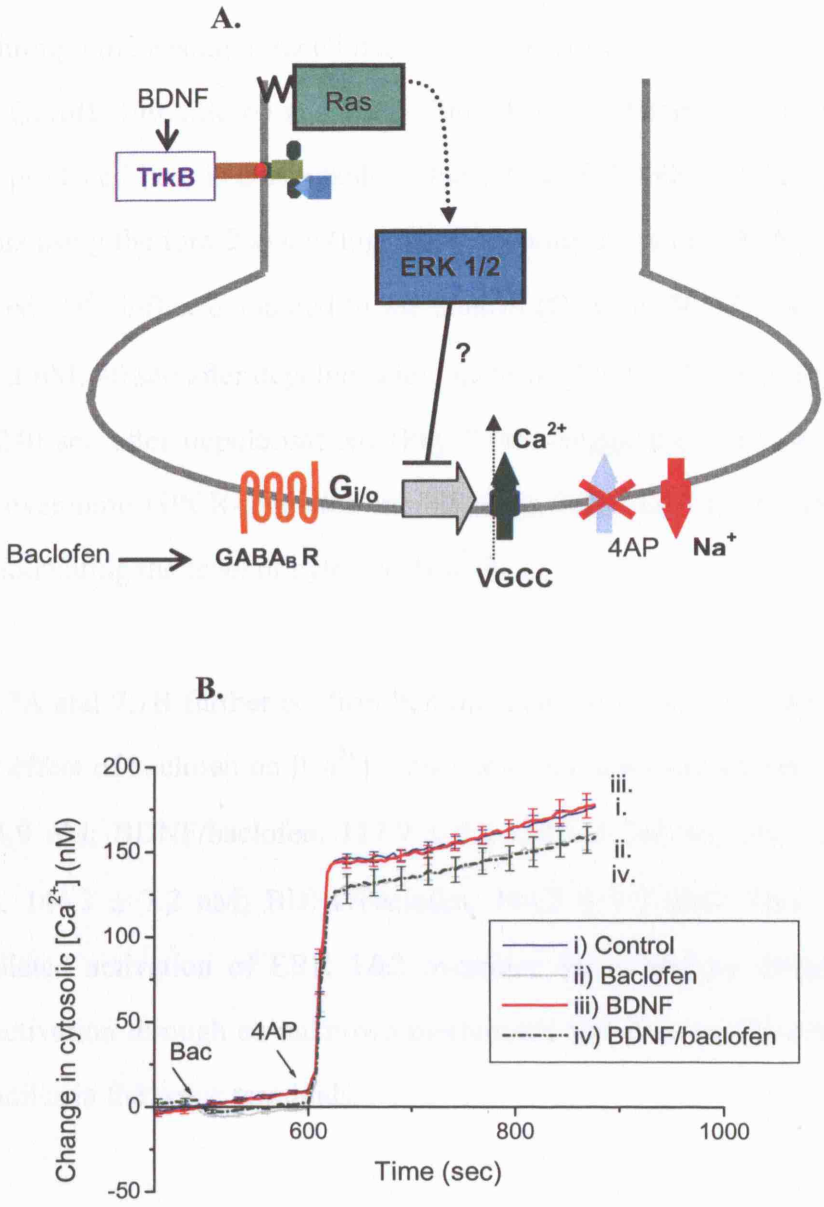



**Figure 7.7. Effect of BDNF and/or baclofen on $Ca^{2+}$ influx induced by secretagogue 4AP.**

**A.** Scheme showing proposed effects of BDNF and/or baclofen on 4AP-induced $Ca^{2+}$ influx. **B.** Fura trace for effects on 4AP-induced changes of cytosolic $Ca^{2+}$ concentrations. Synaptosomes were loaded with fura-2-AM as indicated in the Materials and Methods section. Pellet was then resuspended and incubated in the absence (Control) or presence (BDNF) of 200ng/ml BDNF. Baclofen (50μM) was added before synaptosomes were depolarised with addition of 1mM 4AP at 10 min. Change in cytosolic [$Ca^{2+}$] was calculated from the point of baclofen addition (480 sec). Data represent means ± SEM of experiments carried out with nine (n=9), eight (n=8), six (n=6) and five (n=5) independent synaptosomal preparations for control, BDNF-treated, baclofen-treated and BDNF/baclofen-treated synaptosomes respectively.

Previous studies have suggested that one of the ways in which BDNF can mediate its effect is through increasing intracellular $Ca^{2+}$ levels (Egea et al., 2000; Li et al., 1998; Jiang and Guroff, 1997; Stoop and Poo, 1996). Thus, to determine whether the effect of BDNF produced here is attributable to the effect of BDNF on $[Ca^{2+}]_c$, we directly address this using the fura-2 assay (Fig. 7.6A). Results show that BDNF did not affect 4AP-evoked $Ca^{2+}$ influx compared to the control (Control, 307.7 ± 8.0 nM; BDNF, 304.1 ± 7.2 nM, 40 sec after depolarisation; Control, 326.0 ± 7.1 nM; BDNF, 333.0 ± 8.9 nM, 240 sec after depolarisation) (Fig. 7.6B), suggesting that the neurotrophin does not overcome GPCR-mediated inhibition of ERK 1&2 by $GABA_B$ receptors through modulating the level of cytosolic $[Ca^{2+}]_c$.

Figures 7.7A and 7.7B further confirm that the addition of BDNF does not affect the inhibitory effect of baclofen on $[Ca^{2+}]_c$ either at 40 sec after depolarisation (Baclofen, 119.8 ± 4.9 nM; BDNF/baclofen, 117.9 ± 8.6 nM) or 240 sec after depolarisation (Baclofen, 144.3 ± 7.2 nM; BDNF/baclofen, 144.3 ± 7.7 nM). This confirms that TrkB-mediated activation of ERK 1&2 overrides the inhibitory effect of $GABA_B$ receptor activation through an unknown mechanism that does not involve changes in $Ca^{2+}$ dynamics in the nerve terminals.

## 7.4. Discussion

Presynaptic terminals have a large variety of receptors that can be activated by neurotransmitter released from the same or adjacent terminals. Previous studies have implicated the presynaptic $G_{i/o}$-coupled $GABA_B$ receptors in mediating inhibitory effects on synaptic transmission including neurotransmitter release (Perkinton and Sihra, 1998; Pende et al., 1993; Bonanno and Raiteri, 1992; Potashner, 1979). This chapter assessed the potential crosstalk between the inhibitory $GABA_B$-mediated cascade and the excitatory ERK 1&2 signalling pathway, mechanisms which may underlie the control of neurotransmitter release.

Our results showed that inhibition of ERK 1&2 signalling is provided for by the presence of inhibitory $GABA_B$ receptors in the cerebrocortical nerve terminal. During basal conditions, we showed that the effect of baclofen on basal ERK cannot be readily seen. This was due to the presence of presynaptically "leaked" GABA such that the majority of $GABA_B$ receptors present in the nerve terminals may already be stimulated. By eliminating this tonic inhibition of GABA receptors with exogenous GABase, which metabolises the extrasynaptosomal GABA, we were able to show that basal activities of ERK 1&2 were significantly enhanced. Under these conditions where the effect of endogenous GABA was removed, the basal activated ERK 1&2 was significantly attenuated by the addition of baclofen. The role of $GABA_B$ receptor activation in negatively modulating ERK 1&2 activation was further supported by the ability of baclofen to significantly inhibit depolarisation-induced activation of ERK 1&2. It was interesting to note that the inhibitory effect of baclofen on basal ERK 1&2 activation is not as prominent as that seen with depolarisation-induced activation of ERK 1&2. This is not surprising given that $GABA_B$ receptor activation would be expected to inhibit VGCCs during depolarisation. Previously in chapter 4, we have shown that only 20% of basal ERK 1&2 activities were attributable to $Ca^{2+}$, compared to the large $Ca^{2+}$-dependency of depolarisation-induced ERK 1&2 activation. Indeed,

several studies have suggested that one of the mechanisms by which $GABA_B$ receptors can mediate their inhibitory effects is by affecting $Ca^{2+}$ influx (Perkinton and Sihra, 1998; Dittman and Regehr, 1996), apart from suppressing AC activities or cAMP levels (Sakaba and Neher, 2003; Mott and Lewis, 1994). Furthermore, there is a report showing that the $\alpha 1$ subunit of the N-type VGCCs is a direct target of $GABA_B$ receptor-mediated inhibition (Ikeda and Dunlap, 1999). Here, using fura-2 assay, we showed that baclofen was capable of reducing basal intracellular $Ca^{2+}$ levels. In addition, there is a tendency for baclofen to inhibit depolarisation-induced $Ca^{2+}$ influx, although whether this inhibition was due to the reduction of basal $[Ca^{2+}]_c$ by baclofen was not clear. Given that there is evidence for $GABA_B$ effects to be mediated through inhibiting VGCC activities (Perkinton and Sihra, 1998; Dittman and Regehr, 1996) and that baclofen was incapable of altering membrane potential or ionomycin-induced glutamate release from synaptosomes (Perkinton and Sihra, 1998), it is likely that $GABA_B$ exerts modulation on $Ca^{2+}$-dependent basal and depolarisation-induced ERK activation by affecting $Ca^{2+}$ influx rather than affecting $K^+$ conductance or downstream exocytotic events. Further work using statistical tools such as 2-way ANOVA might be useful to examine the effects of baclofen on depolarisation-induced $Ca^{2+}$ influx more directly, taking into consideration time and condition variables.

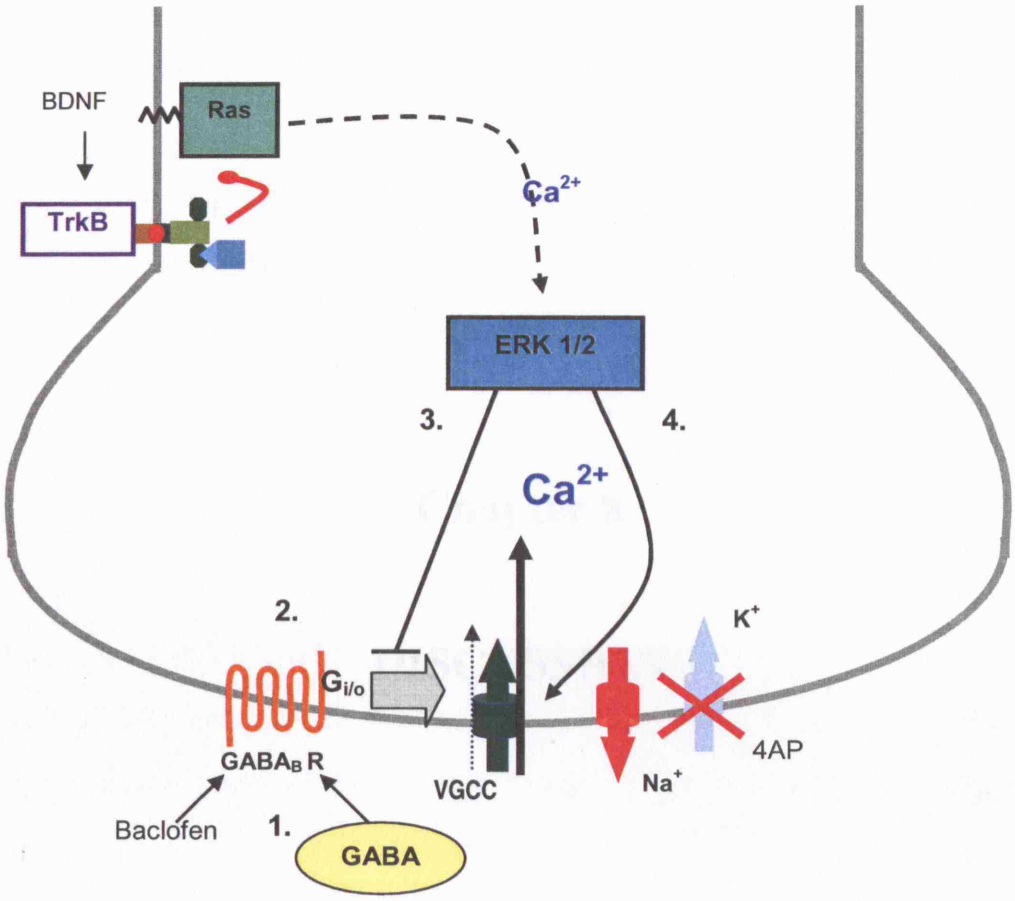
In the present chapter, we provide pharmacological evidence that ERK 1&2 can also modulate presynaptic function by regulating $Ca^{2+}$ dynamics. Data demonstrated that on its own, PD98059, a MEK inhibitor that subsequently blocks the activation of ERK 1&2 (as shown in chapter 3), is able to attenuate 4AP-induced increase in cytosolic $Ca^{2+}$ levels. Thus, unlike $GABA_B$ receptor activation which mediates inhibitory effects on $Ca^{2+}$ influx, the data here suggests that activation of ERK 1&2 activation can lead to events that facilitate $Ca^{2+}$ influx. This might be due to the ability of ERK to modulate $Ca^{2+}$ channel functions by directly phosphorylating the subunits of VGCC (Martin et al., 2006). Having shown previously that ERK 1&2 themselves

are activated by a $Ca^{2+}$-dependent pathway (see Chapter 4), this finding of a positive feedback loop to increase $Ca^{2+}$ influx following ERK activation poses a potential mechanism by which sustained effects of ERK 1&2 activation may be maintained.

Finally, we show that TrkB activation could overcome GPCR-mediated inhibition of ERK 1&2. This was evident by studying the effect of baclofen on ERK 1&2 activation when synaptosomes were stimulated with BDNF. Our data revealed that upon BDNF addition, baclofen has no effect on depolarisation-induced ERK 1&2 activation. This suggests that the facilitatory TrkB-mediated pathway can override the inhibitory pathway and may pose as an important regulatory mechanism during neuronal activity. One possible mechanism for this TrkB-mediated signalling cascade to overcome the $GABA_B$ receptor-mediated inhibitory effects could be through the stimulation of $Ca^{2+}$ influx by TrkB activation. However, unlike previous studies which implicated BDNF in modulating synaptic functions via its ability to regulate intracellular $Ca^{2+}$ levels directly (Egea et al., 2000; Li et al., 1998; Jiang and Guroff, 1997; Stoop and Poo, 1996), data presented here demonstrates that BDNF does not affect basal or depolarisation-induced cytosolic $[Ca^{2+}]$. This lack of effect of BDNF on both basal and depolarisation-evoked elevation of $[Ca^{2+}]_c$ could be attributable to the experimental limitations of measuring $[Ca^{2+}]_c$ in synaptosomes as fura-2 reports only the changes in bulk cytosolic $Ca^{2+}$ rather than localised $Ca^{2+}$ influx (Grynkiewicz et al., 1985). However, given that we have also shown that the addition of BDNF does not affect the ability of baclofen to attenuate depolarisation-induced increase in $[Ca^{2+}]_c$, these data appear to suggest that neurotrophin-mediated signalling overrides inhibitory signalling to ERK 1&2 via an unknown mechanism that does not involve regulation of cytosolic $[Ca^{2+}]_c$ by BDNF. Thus, more experiments are warranted to directly address the exact mechanism by which TrkB-mediated signalling to ERK 1&2 can override the inhibition mediated by $GABA_B$ receptor activation.

In summary, this chapter presented the mechanisms by which the inhibitory $GABA_B$

receptor-mediated pathway can crosstalk with the ERK 1&2 pathway to inhibit their activation in the cerebrocortical nerve terminal. Modulation of these pathways has been proposed to occur at several sites, involving the regulation of $Ca^{2+}$ influx, membrane potential and/or signalling molecules downstream of $Ca^{2+}$ entry. The mechanisms by which ERK 1&2 activation is modulated by the activation of these presynaptic receptors, however, may depend upon the localisation of these receptors as well as other synaptic inputs. Thus, the ability of the nerve terminal to respond to stimuli is a complicated mechanism of crosstalk between excitatory and inhibitory signals. Below is a schematic **(Schematic 7.4)** that illustrates the proposed intracellular signalling pathways underlying the modulation of ERK 1&2 by presynaptic $GABA_B$ receptors.




**Schematic 7.4. The crosstalk mechanism between ERK 1&2 activation and $GABA_B$-mediated signalling pathways.** 1.) $GABA_B$ receptors are activated by ligand GABA or agonist baclofen; 2.) Activation of Gi/o-coupled $GABA_B$ receptor causes inhibition of $Ca^{2+}$ influx through VGCCs, leading to a reduction on basal and depolarisation-induced ERK 1&2 activation; 3.) BDNF-induced activation of ERK 1&2 can overcome the inhibitory effects produced by $GABA_B$ receptor activation; 4.) Activation of ERK 1&2 leads to an increase of $Ca^{2+}$ influx to further strengthen signalling.

# Chapter 8

# DISCUSSION

## 8. DISCUSSION

The ERK 1&2 signalling cascade has been shown to play an important role in the control of synaptic plasticity underlying learning and memory formation (Thomas and Huganir, 2004; Sweatt, 2004). Whilst the vast majority of studies have demonstrated the role of ERK 1&2 in regulating plasticity in the postsynaptic nerve terminal, less is known about their function in the presynaptic compartment. The work in this thesis, thus, examined the activation of ERK 1&2 in the presynaptic nerve terminal and its implication in modulating presynaptic plasticity.

The isolated presynaptic nerve terminal (synaptosome) provides a good model for studying the presynaptic regulatory pathways that lead to ERK 1&2 activation since they are metabolically and functionally competent nerve terminals devoid of the cell body, the axon and the postsynaptic digits (Nicholls and Coffey, 1994; Maycox et al., 1990; Whittaker and Dowe, 1964). Previously, examination of glutamate release from cerebrocortical synaptosomes has indicated a role for ERK 1&2 signalling in the control of synapsin-mediated regulation of exocytosis (Jovanovic et al., 2000). Upstream of exocytosis, activation of ERK 1&2 in the presynaptic component has also been shown to regulate levels of $Ca^{2+}$ influx by directly phosphorylating VGCCs (Martin et al., 2006; Fitzgerald, 2002). This suggests mechanisms by which ERK 1&2 may regulate presynaptic function by altering levels of cytosolic $Ca^{2+}$ in the nerve terminal as well as by modulating activities of synapsin I. Considering these modulatory roles of ERK 1&2 in the presynaptic terminal, the regulation of the ERK 1&2 signalling pathways is, thus, important in the control of synaptic responses following nerve stimulation. Indeed, the present study has found that ERK 1&2 are activity-dependent molecules that are regulated differentially under *basal* and stimulated conditions in the nerve terminals. This thesis has identified two major inputs in the signalling cascade leading to the activation of presynaptic ERK 1&2: i) signalling through the neurotrophin BDNF-mediated pathway that is Ras-dependent;

ii) a $Ca^{2+}$-dependent activation of ERK 1&2 via CaM, CaMKII, SFK and PI3K, all acting as $Ca^{2+}$ signal transducers. In addition, factors that affect the duration and efficacy of ERK 1&2 activation are shown to include protein phosphatases and crosstalk signalling from inhibitory $GABA_B$-receptors.

First and foremost, we have identified the components of the ERK 1&2 signalling pathway activated by the neurotrophin BDNF. The original basis of this work came from previous evidence that BDNF was capable of enhancing depolarisation-induced and ionomycin-induced glutamate release, which was thought to involve ERK 1&2 activation and the subsequent ERK 1&2-dependent phosphorylation of synapsin I (Jovanovic et al., 2000). This TrkB/ERK-dependent signalling cascade was confirmed in our study with the use of a glutamate release assay as well as performing immunochemical studies with activation/phosphorylation-specific antibodies against ERK 1&2. Consistent with Jovanovic *et al.*, we showed that application of exogenous BDNF potentiated 4AP-induced release of glutamate from cerebrocortical synaptosomes. In addition, there was a parallel increase in the phosphorylation of ERK 1&2 in synaptosomes following BDNF stimulation. This suggests that ERK 1&2 is, indeed, a downstream effector of BDNF following TrkB activation that may serve to regulate presynaptic functions. However, despite the fact that this neurotrophin-mediated signalling to ERK 1&2 is one of the best-characterised intracellular signalling pathways known for the modulation of synaptic plasticity (Kaplan and Miller, 1997; Segal and Greenberg, 1996), the mechanisms involved in the regulation of this pathway in the presynaptic nerve terminal remained to be examined.

Here, using the small GTP-binding protein Ras inhibitor, lovastatin, we showed that activation of Ras is involved in this BDNF-mediated activation of ERK 1&2. Furthermore, results also indicated that the addition of lovastatin significantly attenuates the depolarisation-induced release of glutamate, suggesting Ras as an

important player in the regulation of presynaptic neurotransmitter release. These data are consistent with previous studies showing a high level of Ras-GTP present in the nerve terminal (Davies and Sihra, personal communication). In addition, the role of Ras in the BDNF-mediated activation of ERK 1&2 agrees with the existing model of how neurotrophin effects downstream signalling to ERK 1&2 through the recruitment of Shc and Grb2, and the subsequent activation of Ras by Sos (Wellbrock et al., 2004; Huang and Reichardt, 2003; York et al., 1998; Segal and Greenberg, 1996). The activated Ras then mediates activation of the MEK kinases to subsequently activate MEK 1&2, protein kinases immediately upstream of ERK 1&2. The activation of MEK 1&2 by the MEK kinases has, indeed, been shown in our experiment to be essential in the ERK 1&2 signalling pathway with the use of MEK inhibitor PD98059. Thus, both the activation of Ras and MEK 1&2 appear to provide an important link for this neurotrophin-induced pathway leading to ERK 1&2 activation.

With respect to the MEK kinases that are targeted upon Ras activation to translate signals to MEK 1&2, although we have not directly tested this in our preparation, one might speculate on the involvement of the Raf proteins, Raf-1 or B-Raf, given that they are known downstream effectors of Ras (Wellbrock et al., 2004; Hagemann and Rapp, 1999; Bos, 1998). Previously, studies in our lab demonstrated that B-Raf might be the major MEK kinase in the presynaptic nerve terminal since B-Raf was found to be relatively more abundant in the synaptosomes compared to Raf-1 (Davies, 2004). This seems to be consistent with studies which has suggested B-Raf as the more potent activator of MEK than Raf-1 in the brain (Marais et al., 1997). In addition, unlike Raf-1, B-Raf is not subject to inhibition by PKA as the regulatory N-terminal of B-Raf interferes with the ability of PKA to modulate B-Raf activity (MacNicol and MacNicol, 1999). Thus, given that PKA is known to activate ERK 1&2 signalling in synaptosomes (Davies, 2004), this might suggest B-Raf as the likely Ras effector in the presynaptic ERK 1&2 signalling pathway. However, we could not completely rule out the role of Raf-1 since the activation of Raf-1 and B-Raf by Ras is known to be

complex and may involve another member of Ras family, Rap-1 (Wellbrock et al., 2004; Hagemann and Rapp, 1999; Bos, 1998). Thus, the use of Raf-1 and B-Raf inhibitors as well as phosphorylation state-specific antibodies against these Raf proteins will be useful in dissecting their roles in mediating signalling mechanisms that lead to activation of MEK 1&2, and therefore, ERK 1&2 by BDNF.

Independently of the neurotrophin/Ras/MEK-mediated signalling pathway, the present study has demonstrated a role of $Ca^{2+}$ in the activation of ERK 1&2. Our results have implicated $Ca^{2+}$ in the signalling to ERK 1&2 at three possible levels: i) by affecting *basal* activities of ERK 1&2; ii) by affecting depolarisation-induced ERK 1&2 at the level of $Ca^{2+}$ entry; iii) by effecting activation downstream of membrane excitability or VGCC modulation **(Schematic 4.4)**. Under *basal* conditions, ERK 1&2 activities are partially dependent on the presence of $Ca^{2+}$, as suggested by the ~20% inhibition of *basal* activation of ERK 1&2 upon treatment with EGTA. Using the SERCA pump inhibitor thapsigargin, we have shown that this partial $Ca^{2+}$-dependency was mainly attributable to the $Ca^{2+}$ released from intracellular stores. But what causes this release of $Ca^{2+}$ from intracellular stores? One possibility is that under *basal* conditions, activation of phospholipase C (PLC) occurs to mediate $IP_3$ and diacylglycerol production and intracellular $Ca^{2+}$ release from stores. Although this has not been shown directly, this hypothesis seems to be consistent with previous findings in our laboratory that high levels of PLC activity are found in the nerve terminal under *basal* conditions (Coffey et al., 1993; Barrie et al., 1991). More experiments, perhaps, using PLC inhibitors are required to confirm whether this is the case.

Alternatively, $Ca^{2+}$ influx may occur during *basal* conditions to cause a $Ca^{2+}$-induced $Ca^{2+}$ release. This possibility was first suggested by fura-2 measurements which showed a steady rise in $Ca^{2+}$ concentration in the synaptosomes during unstimulated conditions. Indeed, using the blockade of R-type VGCC with the channel toxin, SNX-482 or a knockout of the R-type VGCC, the present study has demonstrated that $Ca^{2+}$

influx via the perisynaptically located R-type VGCCs may contribute to supporting *basal* ERK 1&2 activation. This seems to correlate with findings from other laboratories that R-type VGCCs are involved in modulating spontaneous release in the absence of any exogenous stimulation (Kamp et al., 2005; Watanabe et al., 2004; Gasparini et al., 2001). The $Ca^{2+}$-induced $Ca^{2+}$ release following $Ca^{2+}$ influx via the R-type VGCCs appears to agree with the similar levels of inhibition produced by the blockade of the R-type VGCCs and by thapsigargin. Thus, the dependence of $Ca^{2+}$ in supporting *basal* activation of ERK 1&2 seems to be contributed by $Ca^{2+}$ released from internal stores where this release may be potentially triggered following R-type VGCCs activation. However, more experiments are warranted to directly address this hypothesis.

Contrary to the dependence of *basal* ERK 1&2 activation on intracellular $Ca^{2+}$, we found that ERK 1&2 activation induced following depolarisation was not sensitive to either thapsigargin or R-type VGCC blockade. This was further confirmed by the insignificant effect of the intrasynaptosomal $Ca^{2+}$ chelator BAPTA-AM on 4AP-induced activation of ERK 1&2, suggesting that activation of depolarisation-induced signalling to ERK 1&2 was different to that induced under *basal* conditions with respect to the source of the activating $Ca^{2+}$. Indeed, our results have indicated that depolarisation-induced increase of ERK 1&2 activation is entirely dependent on the presence of $Ca^{2+}$ with the source of this $Ca^{2+}$ being the extrasynaptosomal $Ca^{2+}$ influx through VGCC following depolarisation. Thus, a $Ca^{2+}$-dependent mechanism appears to exist whereby levels of ERK 1&2 activation can be altered to fit the requirement of the nerve terminal in response to changes in $Ca^{2+}$ levels that is ultimately determined by the levels of presynaptic activities.

Studies have shown that ERK 1&2 may further strengthen the $Ca^{2+}$-dependent signal by modulating the activities of VGCCs (Martin et al., 2006). Consistent with this, our data have shown that VGCCs could be regulated by ERK 1&2 to affect synaptic

response to depolarisation in the presynaptic nerve terminal. This was shown using the MEK inhibitor PD98059 which significantly attenuated 4AP-induced $Ca^{2+}$ influx into the synaptosomes. Downstream of modulation at the level of membrane excitability and VGCCs, we confirmed the $Ca^{2+}$-dependency of ERK 1&2 activation directly with the use of $Ca^{2+}$ ionophore ionomycin. By titrating intrasynaptosomal levels of $Ca^{2+}$ with added cation in the presence of ionomycin, our results showed that ERK 1&2 activation is elevated upon the addition of buffered free $Ca^{2+}$. These data indicate that ERK 1&2 can be activated by $Ca^{2+}$ at a level downstream of modulation at the plasma membrane.

With respect to the $Ca^{2+}$-dependent signalling to ERK 1&2, an immediate question arises as to what are the $Ca^{2+}$ sensors or mediators for the $Ca^{2+}$-dependent ERK 1&2 pathway. Although there is no evidence for the direct binding and/or activation of ERK 1&2 itself, studies have reported a number of ways by which $Ca^{2+}$ can affect ERK 1&2 signalling by acting upon upstream regulators such as Ras-GRF and Ras-GEF (Aspenstrom, 2004; Walker et al., 2003; Farnsworth et al., 1995). In some cases, $Ca^{2+}$ has been shown to activate the K-isoform of Ras directly, although this is thought to result in decreased ERK 1&2 signalling (Villalonga et al., 2001). Having demonstrated Ras as an essential regulator in the BDNF-mediated ERK 1&2 signalling cascade, this might suggest that $Ca^{2+}$ can also impinge on the ERK 1&2 pathway at the level of Ras activation. Indeed, we have found that the $Ca^{2+}$-dependent activation of ERK 1&2 under *basal*, depolarisation, as well as ionomycin/$Ca^{2+}$-induced conditions, are sensitive to the Ras inhibitor lovastatin, clearly indicating that these $Ca^{2+}$-dependent mechanisms work to cause an increase in ERK 1&2 activities via the activation of Ras. Notwithstanding, other studies have also shown that $Ca^{2+}$-dependent regulation of ERK signalling can be exerted at the level of Raf-1, downstream of Ras (Egea et al., 2000; Egea et al., 1999). This issue may be addressable with future experiments using newly developed Raf-1 inhibitors (Lackey et al., 2000).

In the present study, we have shown a number of $Ca^{2+}$ signal transducers that can relay signals to stimulate the activation of ERK 1&2 in the synaptosomes. Using antagonists that inhibit CaM (W7) and CaMKII (KN93), we were able to show the role of the $Ca^{2}$-sensing protein CaM and its protein kinase effector CaMKII in mediating *basal* and $Ca^{2+}$-dependent activation of ERK 1&2. Previous studies have shown that CaMKII may act to phosphorylate SynGAP, a GAP for Ras, to subsequently mediate signalling to ERK 1&2 (Oh et al., 2004; Bos, 1998; Chen et al., 1998). Alternatively, CaMKII dependent phosphorylation may impinge directly on Raf-1 to modulate activation of ERK 1&2 (Illario et al., 2003). However, modulation at the level of Raf-1 by CaMKII appears unlikely since, using lovastatin, we have shown that $Ca^{2+}$-dependent signalling to ERK 1&2 is contingent on the activation of Ras, which is upstream of Raf-1. Experiments using KN93 to examine its effect on the activation of Ras may help to confirm the involvement of CaMKII either at the level of Ras activation or downstream. Activation of Ras could be measured using immunoprecipitation assay with Raf binding domain-containing glutathione sepharose beads that bind to activated Ras.

Another possible mechanism by which CaMKII may mediate $Ca^{2+}$-dependent signalling to ERK 1&2 in the presynaptic nerve terminal is through the activation of SFKs. Previously, this mechanism of ERK 1&2 activation has been proposed in vascular smooth muscle (Ginnan and Singer, 2002). Consistent with the study by Ginnan *et al.*, we showed that ionomycin/$Ca^{2+}$-induced activation of ERK 1&2 was attenuated by the addition of the SFK inhibitor PP2 as well as by KN93. In addition, we have demonstrated that inhibition of SFK function was associated with a decrease in depolarisation-induced glutamate release with the PP2 inhibitor, but not the inactive analogue PP3, indicating that activation of ERK 1&2 is dependent on the role of SFKs and that this may possibly underlie the modulation of presynaptic function. Thus, these results seem to suggest that the $Ca^{2+}$-dependent activation of ERK 1&2 in

the presynaptic nerve terminal may occur through a pathway that involves both the activation of CaMKII and SFKs. However, whether $Ca^{2+}$-dependent signalling to ERK 1&2 is mediated through CaMKII-dependent activation of SFKs following an increase in intracellular $Ca^{2+}$ remains to be addressed. The use of KN93 to study tyrosine phosphorylation of SFKs will be useful in determining whether SFK activation take places before or after CaMKII activation.

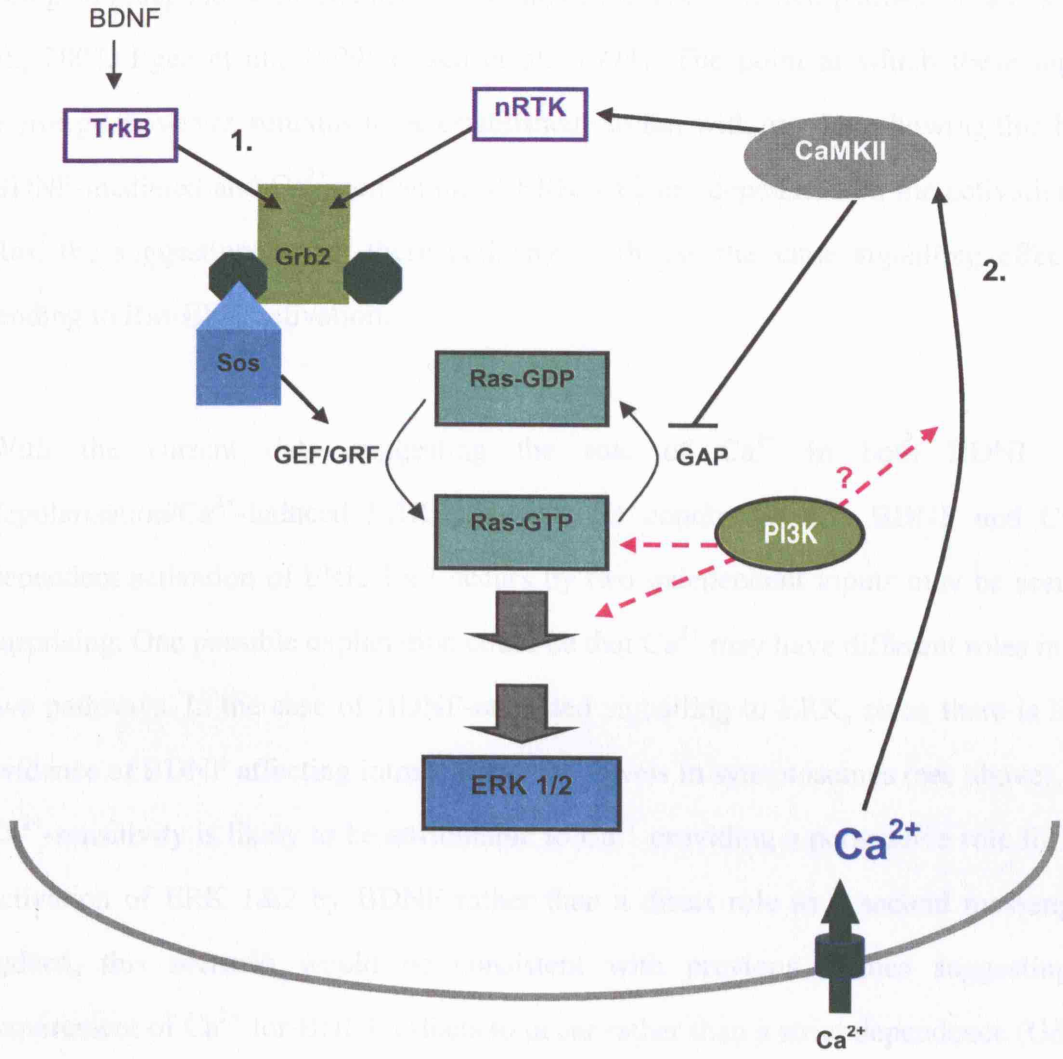
With regards to the action of SFKs on the $Ca^{2+}$-dependent signalling to ERK, we have demonstrated that *basal* and depolarisation-induced ERK 1&2 were both inhibited by PP2 to similar levels obtained using the Ras inhibitor lovastatin. This seems to suggest that $Ca^{2+}$-dependent ERK 1&2 signalling is contingent on both the activation of Ras and SFKs in a pathway where SFKs are likely to act upstream of Ras. One possibility is that SFKs may mediate $Ca^{2+}$-dependent signalling to ERK 1&2 by modulating neuronal excitability to increase intracellular $Ca^{2+}$ levels, and thereby, increase ERK 1&2 activation. Indeed, previous studies have found that SFKs can directly increase $Ca^{2+}$ influx via VGCCs in synaptosomes (Wang, 2003; Evans and Pocock, 1999). However, given that our data demonstrated that ionomycin-induced activation of ERK 1&2 is also attenuated by PP2, the action of SFKs appears to be downstream of $Ca^{2+}$ entry. In this regard, there are two possible targets which SFKs may act upon to mediate signals to ERK 1&2: i) by acting as a scaffold for the assembly of ERK signalling regulators such as Shc and Grb2 to allow activation of Sos (Crossthwaite et al., 2004; Rocic et al., 2001; Luttrell et al., 1996); ii) by interacting directly with upstream modules of ERK 1&2 signalling cascade including the TrkB receptors (Iwasaki et al., 1998) and Ras (Kalia et al., 2004). Future experiments using immunoprecipitation techniques may be able to dissect whether SFKs are involved in directly associating with Shc, Grb2, Ras or TrkB to lead to ERK 1&2 activation. If the hypothesis that CaMKII-mediated activation of SFK is true, KN93 should reduce the function of SFK itself or its interaction with upstream modules of ERK signalling.

Recently, studies in cultured striatal neurons have demonstrated that $Ca^{2+}$/SFK-dependent signalling to ERK 1&2 is contingent on the activation of PI3K as an intermediate signalling molecule (Crossthwaite et al., 2004; Perkinton et al., 2002). Consistent with these studies, we showed that PI3K was involved in mediating the $Ca^{2+}$-dependent ERK 1&2 activation pathway with the PI3K inhibitor LY29004. The role of PI3K in the presynaptic function was further confirmed by its involvement in 4AP-induced glutamate release. Taken together, these results suggest that PI3K may act in the nerve terminal to modulate the $Ca^{2+}$-dependent signalling to ERK 1&2. PI3K may exert control on the ERK 1&2 cascade either upstream or downstream of Ras activation. Previous studies have indicated that PI3K may have a permissive role in ERK 1&2 activation by inhibiting Ras-GAP through the production of 3-phosphoinositides, and in so doing, affect the accumulation of Ras in the cell (Rubio and Wetzker, 2000). Alternatively, downstream of Ras, PI3K may mediate ERK 1&2 signalling by regulating the activities of Raf-1 or MEK (Bondeva et al., 1998; Hu et al., 1995). This is consistent with the studies showing that activation of PI3K can occur following Ras activation (Rommel et al., 1999; Downward, 1998; Rodriguez-Viciana et al., 1994). Considering these possibilities, future experiments looking at the effects of LY29004 on Ras activation will be able to distinguish between the function of PI3K before or after Ras activation.

In the foregoing discussion, ERK 1&2 activation by $Ca^{2+}$ has been suggested to be important in the modulation of presynaptic function and involve the action of various mediators including Ras, CaMKII, SFK and PI3K. Increasingly, there is evidence to suggest that $Ca^{2+}$ also plays a role in neurotrophin-mediated signalling to ERK 1&2. Studies in a number of systems have pointed to the ability of BDNF to modulate synaptic activity by increasing levels of cytosolic $Ca^{2+}$ (Egea et al., 2000; Li et al., 1998; Jiang and Guroff, 1997; Stoop and Poo, 1996). In these studies, BDNF stimulation is proposed to cause elevation of $Ca^{2+}$ by modulating the activities of

VGCCs to affect $Ca^{2+}$ influx (Baldelli et al., 2000; Baldelli et al., 1999) and/or by initiating release of $Ca^{2+}$ from intracellular stores (Li et al., 1998; Stoop and Poo, 1996). Thus, the question arises as to whether BDNF may also function to affect intrasynaptosomal $Ca^{2+}$ levels to effect downstream activation of ERK 1&2. We have directly addressed this by measuring the changes in cytosolic $Ca^{2+}$ following the addition of exogenous BDNF using the fura-2 assay. Our results have shown that BDNF does not affect basal or 4AP-evoked $Ca^{2+}$ influx, suggesting that it is unlikely for BDNF to initiate ERK signalling via the elevation of intracellular $Ca^{2+}$. However, as fura-2 measures global levels of $Ca^{2+}$ rather than localised $Ca^{2+}$ changes, we could not totally exclude the possibility of BDNF affecting changes in $[Ca^{2+}]_i$ in sub-compartments. Given the size of the nerve terminal, such localised increases in $[Ca^{2+}]_i$ in sub-compartments of the nerve terminal are, however, difficult to address.

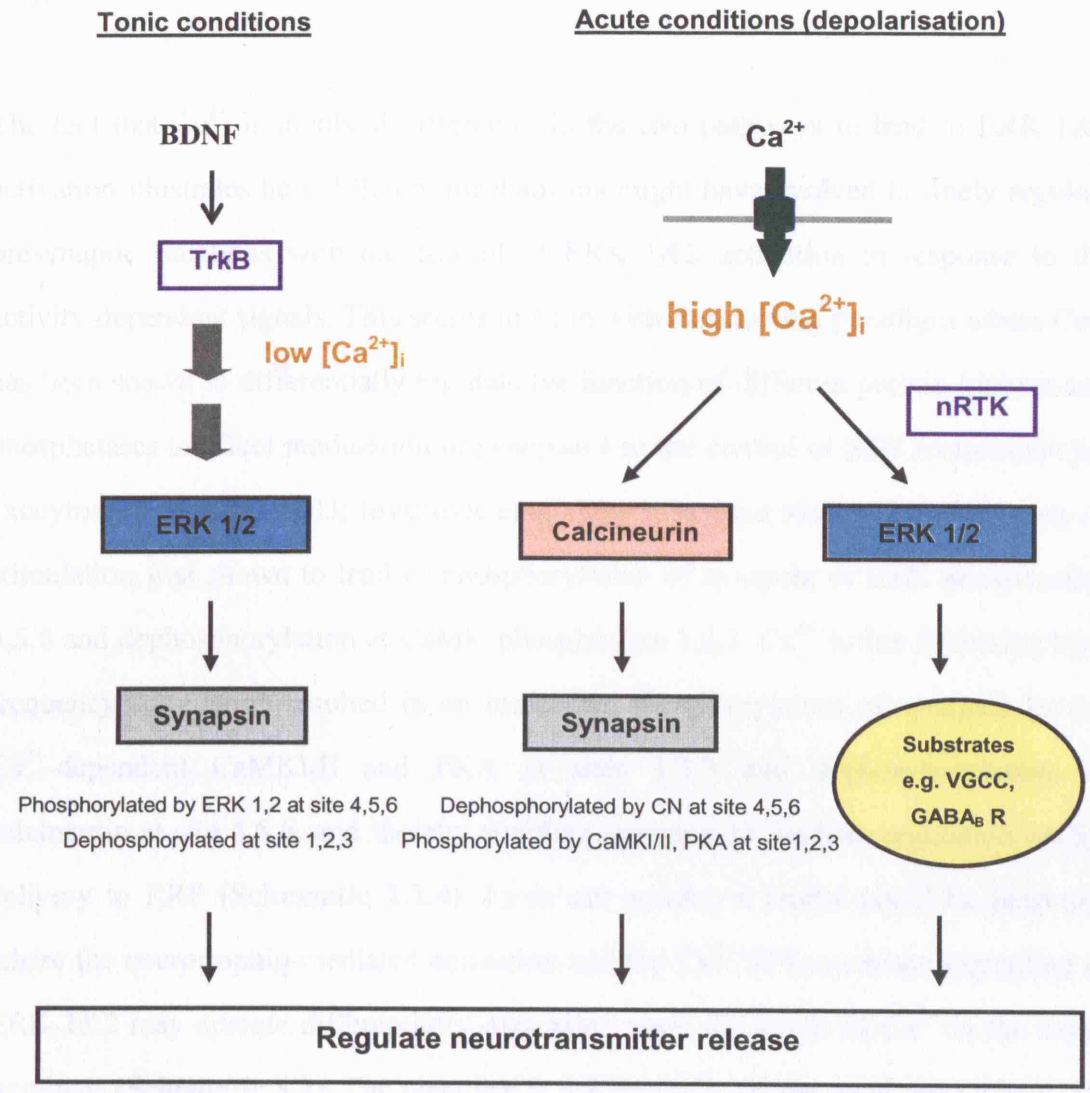
Interestingly, despite apparently not affecting $[Ca^{2+}]_i$, we have demonstrated that BDNF-mediated activation of ERK 1&2 is *sensitive* to the presence of $Ca^{2+}$. Our data have shown that synaptosomal incubation with EGTA resulted in a significant reduction in BDNF-induced ERK 1&2 activation. In addition, with the use of KN93, we found that CaMKII is involved in mediating this $Ca^{2+}$ signal in BDNF-mediated activation of ERK 1&2 in our preparation. Given that both the BDNF-mediated activation of ERK and the $Ca^{2+}$-dependent signalling to ERK 1&2 require the presence of $Ca^{2+}$, the immediate question would be are these inputs sequential and inter-dependent or parallel to each other? The present study suggests that the BDNF-mediated pathway and depolarisation/$Ca^{2+}$-dependent pathway are two independent inputs that feed into a common ERK 1&2 signalling cascade. This is shown by the mutual occlusion of BDNF-mediated and depolarisation/ionomycin-stimulated activation of ERK 1&2. In addition, we showed that inhibition of SFKs with PP2 does not affect BDNF-mediated increase in ERK 1&2 activation, thus, suggesting that the TrkB and the $Ca^{2+}$/SFK -mediated signalling cascade are two inputs that are not contingent on the activation of each other but are distinct inputs leading to a single




**Schematic 8.1. The two independent inputs leading to ERK 1&2 activation in the nerve terminal identified in this thesis.** 1.) BDNF-mediate activation of ERK 1&2 via activation of adaptor Grb2 and Sos to lead to Ras activation. In this pathway, $Ca^{2+}$ act to provide a permissive role for downstream activation to occur; 2.) $Ca^{2+}$-activation of ERK 1&2 via the activation of Ras and involving the $Ca^{2+}$ transducer CaMKII, SFKs and PI3K. Activation of SFK may be mediated following CaMKII activation by acting on adaptor Grb2 or at the level of Ras activation. PI3K may act at the level of Ras by inhibiting Ras-GAP or at the level of Raf-1.

cascade of ERK 1&2 activation downstream. This is consistent with findings from other laboratories that proposed depolarisation/$Ca^{2+}$-induced signalling to ERK as being an independent mechanism from that of the Trk-mediated pathway (Baldassa et al., 2003; Egea et al., 1999; Rosen et al., 1994). The point at which these inputs converge, however, remains to be established. So far, with our data showing that both BDNF-mediated and $Ca^{2+}$-activation of ERK 1&2 are dependent on the activation of Ras, the suggestion is that these pathways both use the same signalling effectors leading to Ras-ERK activation.

With the current data suggesting the role of $Ca^{2+}$ in both BDNF and depolarisation/$Ca^{2+}$-induced ERK pathway, the conclusion that BDNF and $Ca^{2+}$-dependent activation of ERK 1&2 occurs by two independent inputs may be seen as surprising. One possible explanation could be that $Ca^{2+}$ may have different roles in the two pathways. In the case of BDNF-mediated signalling to ERK, since there is little evidence of BDNF affecting intracellular $Ca^{2+}$ levels in synaptosomes (see above), the $Ca^{2+}$-sensitivity is likely to be attributable to $Ca^{2+}$ providing a permissive role for the activation of ERK 1&2 by BDNF rather than a direct role as a second messenger. Indeed, this scenario would be consistent with previous studies suggesting a requirement of $Ca^{2+}$ for BDNF effects to occur rather than a strict dependence (Goggi et al., 2002; Kang and Schuman, 2000; Stoop and Poo, 1996). Thus, under conditions where normal $[Ca^{2+}]_i$ is present, $Ca^{2+}$ might enable the activation of CaMKII to mediate inhibition on Ras-GAP, such that BDNF stimulation of ERK 1&2 signalling may take place more effectively with the increased Ras-GEF or GRF activities. However, when the intrasynaptosomal $Ca^{2+}$ is absent under experimental conditions, the constitutive inhibition of Ras-GAP by CaMKII would no longer occur, so that the now strong activity of GAP would tend to counteract any effective production of Ras-GTP through BDNF-mediated stimulation of Ras-GEF. Thus, $Ca^{2+}$ might appear to play a permissive role in BDNF-mediated signalling to ERK 1&2 activation. In the case of depolarisation or ionomycin/$Ca^{2+}$-induced ERK 1&2 signalling, however,




**Schematic 8.2. A proposed model for the activation of ERK 1&2 in the presynaptic nerve terminal under tonic and acute conditions.** Under conditions when intrasynaptosomal $Ca^{2+}$ is low, BDNF-mediated activation of ERK 1&2 occurs, such that subsequent phosphorylation of synapsin at phosphosites 4,5 by ERK takes place to regulate neurotransmitter release. At acute conditions, $Ca^{2+}$ influx following depolarisation induces activation of ERK 1&2 to phosphorylate other substrates such as VGCCs and $GABA_B$ receptors to further increase synaptic strength. The $Ca^{2+}$-dependent calcineurin was activated in response to $Ca^{2+}$ influx to dephosphorylate synapsin at sites 4,5,6 whilst CaMKI/II and PKA phosphorylate synapsin at sites 1,2,3.

$Ca^{2+}$ may play a more direct function as a second messenger to transduce signals to activate ERK 1&2 via a pathway that involve CaMKII, SFK and PI3K **(Schematic 8.1)**.

The fact that $Ca^{2+}$ is involved differently in the two pathways to lead to ERK 1&2 activation illustrates how different mechanisms might have evolved to finely regulate presynaptic functions with the control of ERK 1&2 activation in response to the activity-dependent signals. This seems to fit in with the existing paradigm where $Ca^{2+}$ has been shown to differentially regulate the function of different protein kinases and phosphatases to effect modulation of synapsin I in the control of SSV recruitment for exocytosis (Chi et al., 2003; Jovanovic et al., 2001). In these studies, low frequency of stimulation was shown to lead to phosphorylation of synapsin at ERK phosphosites 4,5,6 and dephosphorylation at CaMK phosphosites 1,2,3. $Ca^{2+}$ influx following high frequency stimulation resulted in an increasing phosphorylation of synapsin by the $Ca^{2+}$-dependent CaMKI/II and PKA at sites 1,2,3 and dephosphorylation by calcineurin at site 4,5,6, and thereby enabling synapsin to mediate regulation on SV delivery to RRP **(Schematic 1.2.4)**. From our results, a model could be proposed where the neurotrophin-mediated activation and the $Ca^{2+}$/SFK-mediated signalling to ERK 1&2 may operate differentially depending upon the levels of $Ca^{2+}$ in the nerve terminals **(Schematic 8.2)**. The postulate is that tonically, under conditions where low cytosolic levels of $Ca^{2+}$ are present, $Ca^{2+}$ may play a permissive role for BDNF-mediated activation of ERK 1&2 to occur (see above), such that the activated ERK 1&2 then enables synapsin to be phosphorylated at site 4,5 whilst synapsin sites 1,2,3 are dephosphorylated. During depolarisation, $Ca^{2+}$ influx via VGCCs may activate ERK 1&2 to phosphorylate substrates such as VGCCs (Martin et al., 2006) and $GABA_B$ receptors (see chapter 7), both of which may lead to increase in synaptic strength. During such acute conditions, CaMKI/II and PKA would phosphorylate synapsin on sites 1,2,3, while the increased $Ca^{2+}$ concentrations in the nerve terminal would activate the $Ca^{2+}$-dependent calcineurin to trigger dephosphorylation of

synapsin at site 4,5,6.

Having considered the possible mechanisms by which BDNF-mediated and the $Ca^{2+}$/SFK-mediated cascades may be involved in activating presynaptic ERK 1&2 under tonic and acute conditions respectively, the question remaining as to the factors that may affect the duration and efficacy of ERK 1&2 activation.

Firstly, we have evaluated the protein phosphatases involved in regulating the *basal* activities of ERK 1&2 in our preparation. Using a general PP1 and PP2A inhibitor, okadaic acid, we showed that addition of the drug results in the potentiation of *basal* ERK 1&2 activation in a dose-dependent manner. Given that we were unable to show any ERK modulation by the $Ca^{2+}$-dependent phosphatase calcineurin and that the involvement of PP1 in presynaptic function has been previously questioned (see chapter 3), the indication is that PP2A is the likely phosphatase exerting negative control on *basal* ERK 1&2 activities. Although we have not identified the level at which PP2A impinges to inhibit ERK 1&2 signalling, one might speculate that PP2A may act on ERK 1&2 directly since there is evidence that PP2A can dephosphorylate ERK 1&2 (Van Kanegan et al., 2005). Alternatively, PP2A may regulate ERK 1&2 indirectly via a mechanism involving MEK1 &2 (Mao et al., 2005). Whether PP2A exerts control at the level of Ras and/or Raf-1 activation to mediate inhibition on ERK 1&2 signalling remains uncertain. More experiments are therefore warranted to examine the precise target(s) of PP2A in mediating ERK 1&2 inhibition. The use of the Ras inhibitor lovastatin and/or the MEK inhibitor PD98059 on okadaic acid-induced increase of ERK 1&2 might be helpful in dissecting the involvement of PP2A before or after Ras activation.

Secondly, using the cdk5 inhibitor roscovitine, we have shown that cdk5 is also involved in negatively modulating ERK 1&2 activation under *basal* conditions. Indeed, the inhibitory role of cdk5 has been demonstrated previously using p35

knockout mice where cdk5 has been found to downregulate ERK 1&2 by decreasing activities of Ras-GRF and/or MEK (Kesavapany et al., 2004; Sharma et al., 2002). However, during stimulation, our results showed that roscovitine inhibited rather than enhanced 4AP-induced and BDNF-stimulated ERK 1&2 activation. Whilst this might suggest that cdk5 plays a different role in facilitating rather than inhibiting ERK 1&2 signalling following stimulation, more likely, the data demonstrates that stimulation of the nerve terminal by depolarisation or by neurotrophin addition may override the cdk5-mediated inhibition of ERK 1&2. One possible experiment to perform to test this is with the use of antibodies against cdk5 to detect changes in the level of activated cdk5 under *basal* and stimulated conditions and compare this with activation of ERK 1&2. Experiments using ionomycin to bypass modulation at the level of VGCCs will also address the specificity of roscovitine as cdk5 inhibitor in our preparation as this has been of issue in previous studies (Yan et al., 2002; Tomizawa et al., 2002).

Finally, our results have also indicated that inhibition of ERK 1&2 occurs due to the presence of inhibitory $G_{i/o}$-coupled $GABA_B$ receptors in the synaptosomes. We have shown that, even under *basal* conditions, there was a 'background' of endogenously released GABA present that caused a stimulation of the presynaptic $GABA_B$ receptors in the nerve terminal to inhibit activation of ERK 1&2. When this endogenous GABA was removed, the addition of the agonist baclofen demonstrated that $GABA_B$ activation could attenuate ERK 1&2 activities. In addition, we have shown that depolarisation-induced activation of ERK 1&2 was also significantly reduced by baclofen. These data suggest that $GABA_B$ receptor activation may have a role in modulating both *basal* and depolarisation-induced activities of ERK 1&2. Consistent with studies showing that $GABA_B$ receptor activation is associated with the downregulation of $Ca^{2+}$ influx via VGCCs (Perkinton and Sihra, 1998; Dittman and Regehr, 1996), we were able to show that baclofen can reduce basal $Ca^{2+}$ levels in our preparation by assessing intrasynaptosomal $Ca^{2+}$ levels using fura-2. In addition,

there is a tendency for baclofen to reduce depolarisation-induced $Ca^{2+}$ influx, although this requires further examination using statistical methods. Notwithstanding other findings of modulation of AC activities (Fairfax et al., 2004; Sakaba and Neher, 2003) or the activation of Kv channels (Kubota et al., 2003; Misgeld et al., 1989) following $GABA_B$ receptor activation, this suggests a signalling pathway by which $GABA_B$ receptor mediates its inhibitory effect on ERK 1&2 signalling by regulating the activities of VGCCs. Furthermore, we have shown that the effect of $GABA_B$ receptors suppressing ERK 1&2 activation could be overcome by activation of the TrkB receptors. This is demonstrated with the addition of BDNF, when baclofen has no apparent effect on ERK 1&2 activation in the presence of the exogenously applied neurotrophin. More experiments may be required to confirm the level at which this reciprocal crosstalk take place between the facilitatory TrkB-mediated ERK 1&2 activation and the inhibitory $GABA_B$-mediated pathway. Eludiation of whether $GABA_B$-mediated inhibitory effect is exerted before or after Ras activation using baclofen may be helpful.

In conclusion, I have presented evidence that the ERK 1&2 activation pathway is a highly regulated signalling cascade in the presynaptic nerve terminal, involving various modulators that act in combination to either enhance or inhibit their activities. Our results have identified two major inputs for the activation of ERK 1&2 in the nerve terminal: i) neurotrophin-mediated ERK 1&2 signalling via a Ras-dependent pathway; ii) $Ca^{2+}$-activation of ERK 1&2 that involves the function of Ras, CaMKII, nRTK and PI3K. In addition, the efficacy and duration of ERK 1&2 activation has been shown to be modulated by the activation of PP2A and cdk5 as well as through $GABA_B$ receptor-mediated signalling. Central to the regulation of ERK 1&2 by these modulators is the presence of $Ca^{2+}$ which has established important gateways for ERK 1&2 to be regulated specifically and differentially in response to changing synaptic activities, and thereby, contributes to the kinases' ability to modulate presynaptic plasticity.

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